Platelets in infectious diseases: the silent force

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"...and what is done in love is well done."
- Vincent van Gogh

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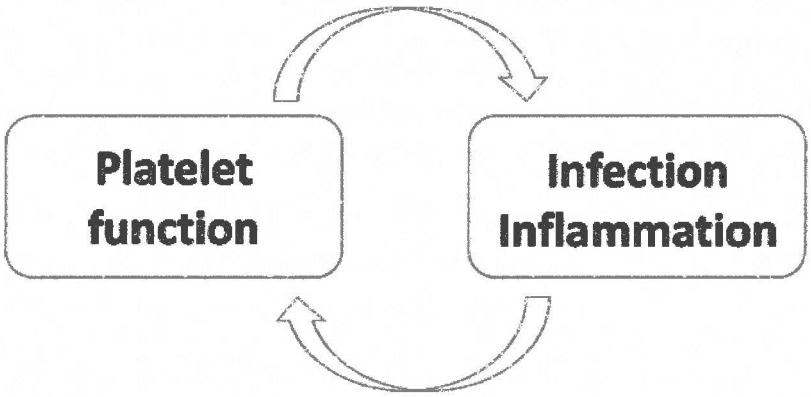
Chapter 1

General introduction

General Introduction

Platelets are traditionally known for their role in primary hemostasis, a process in which platelet plugs seal off an injury to a blood vessel to prevent further bleeding. Platelets also play a fundamental role in progression of atherosclerosis and acute cardiovascular events (1). Beyond these apparent functions in hemostasis and thrombosis, it is increasingly recognized that platelets are key cells in immunity and inflammation. Platelets can interact with different immune cells and alter their phenotype (2). Platelets can also release antimicrobial peptides (3) and trigger the formation of neutrophil extracellular traps (4), highlighting their involvement in the innate host response against infectious diseases. Infection/inflammation and hemostasis are thus two processes which interact with each other, and platelets play a crucial role in this bidirectional interaction.
Severe infections and the related changes in inflammation may have differential effects on platelets. High platelet numbers are commonly seen in infectious diseases such as tuberculosis (5), whereas low platelet counts are a feature of viral infections, such as dengue (6). The changes in platelet numbers and function may not only be a result of inflammation, but also be a direct or indirect effect of the micro-organisms itself. The present research mainly focuses on how infection and inflammation alter platelet numbers and function, and whether and how these platelet changes alter the innate immune response against these infections.



Platelet structure, lifespan and reactivity
Platelets have an individual lifespan of 8-10 days, with 100 billion platelets produced daily by megakaryocytes to maintain the normal count of 150–400 × 10^9 platelets per liter of blood. Although small in size (with a diameter of 1.5-3 μm), they are present in large numbers in the circulation. Platelets have a large surface area due to an open canalicular system which can take up proteins and molecules and re-release them upon activation. Platelets contain 3 principal types of granules: the α-granules, dense

granules and lysosomes. These granules store over 300 biologically active molecules which can be released to the circulation or translocated to the platelet membrane during platelet activation (2). The platelet α-granules are the most abundant (50-80 per platelet) and contain a large variety of biologically active substances such as membrane proteins (αIIbβ3 integrin, P-selectin and CD36), adhesive molecules (fibrinogen, von Willebrand factor [VWF], vitronectin and multimerin 1), coagulant factors (factor V, IX and XIII) and chemokines with direct microbicidal properties. Platelet α-granules are also participating actively in inflammation due to the secretion of pro-inflammatory and immune-modulating factors (7). P-selectin (CD62P) is a platelet α-granule transmembrane protein which is expressed on the surface membrane after an activation signal and interacts with its counter-ligand, P-selectin glycoprotein ligand-1 (PSGL-1), mediating the interaction between platelets and leukocytes (8). The platelet dense granules (3-8 per platelet) contain high concentration of calcium, phosphate and hemostatic agonists such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), whereas platelet lysosomes contain acid hydrolase, cathepsin and lysosomal proteins (2).

Platelets have a diverse array of receptors that trigger their activation upon exposure to hemostatic agonists or subendothelial matrix. The initial tethering of platelets at sites of vascular injury is mediated by the glycoprotein (GP) Ib/V/IX, a structurally unique receptor complex expressed by platelets. One of the component of this complex, GPIb, interacts with VWF as its major ligand (9). Besides GPIb, other collagen receptors for platelet tethering are present on the platelet surface, including GPVI and GPIa/IIa (or α2β1 integrin), members of the immunoglobulin superfamily (10). Activated platelets release arachidonic acid from membrane phospholipids, which is subsequently converted to thromboxane A2 (TxA2). As a consequence, TxA2 is secreted and acts on other platelets through the TP receptors, which belong to the major receptor family G-protein coupled receptors (GPCRs), leading to further platelet activation (11). After activation, platelets undergo rapid change of shape and release their granule content. One soluble platelet agonist released from dense granules is ADP, which activates platelets through their $P2Y_1$ and $P2Y_{12}$ receptors (12). Thrombin, another platelet agonist, binds another GPCR named protease-activated receptors (PARs). Thrombin-mediated platelet activation in humans occurs through the activation of PAR-1 and PAR-4 (13). Additionally, platelet activation causes a conformational change in αIIbβ3 integrin, enabling the binding of fibrinogen, which form bridges between adjacent platelets and facilitate platelet aggregation (13). Thus, platelet activation is the product of many signals from different receptors, which each contribute to the formation of a platelet plug.

Platelets as immune cells: the crosstalk with immunity

Platelets play important roles in innate immunity by participating in antimicrobial host

defense, leukocyte trafficking and immunothrombosis (14). Platelets can interact with immune cells such as monocytes, neutrophils, dendritic cells and memory T cells, and as a result alter their phenotypes. Among all leukocytes, monocytes show the highest affinity for platelets (15). This platelet-monocyte crosstalk triggers the release of pro-inflammatory cytokines, monocyte recruitment and extravasation, monocyte CD16 expression and a more pro-coagulant phenotype (16-19). However, this crosstalk can also switch monocytes into an anti-inflammatory phenotype, for example by upregulating of IL-10 and suppressing TNF-α and IL-6 responses to lipopolysachharide (LPS), thyroglobulin or *Porphyromonas gingivalis* (20).

Toll-like receptors (TLRs) form an important part of host defense against invading pathogens by recognition of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Platelets also harbor different TLRs and different studies have confirmed the functionality of both platelet surface TLRs (TLR1, TLR2, TLR4 and TLR6) and intracellular TLRs (TLR3, TLR7, TLR9) (reviewed by (21)). TLR2 and TLR4 are predominantly known to recognize common cell wall components of Gram-positive and Gram-negative bacteria, respectively. TLR2 ligates peptidoglycan and lipoteichoic acid bacterial cell wall components of Gram-positive bacteria (22), whereas LPS from Gram-negative bacteria is recognized by TLR4 (23). Pam3CSK4, a synthetic ligand of TLR2, has been shown to have strong thrombotic platelet activating properties, in contrast to LPS (24). In addition to innate immune responses, platelets are also recognized to be involved in adaptive immunity. For example, platelets facilitate lymphocyte homing, immunoglobulin class switch, and germinal center formation (25, 26).

Platelet function assays

Unlike data on changes in platelet number, which can be easily monitored, surprisingly few data are available on the effect of infectious diseases on platelet reactivity. The limited availability of reliable analytical assays to capture *in vivo* platelet function and the fact that platelet samples need to be processed immediately without prior storage are likely to account for this. The traditional method of platelet function test utilizes light transmission aggregometry. This test performs poorly when patients are thrombocytopenic and requires prior isolation of platelet-rich plasma. Another option is to measure platelet activation and the platelet response to *ex vivo* activation by different platelet ligands using flow cytometry. Recently, our collaborators developed a whole blood, flow cytometry-based platelet reactivity assay (27), which has the advantages that blood manipulation to produce PRP is not required and that it can be employed in thrombocytopenic conditions. Readout of this assay is the expression of the α-granule protein P-selectin and activation of the αIIbβ3 integrin by measuring the binding of fibrinogen. This is measured in unstimulated samples and after ex vivo stimulation with

different platelet agonists such as collagen-related peptide (CRP-XL), thrombin receptor activating peptide (TRAP) and adenosine diphosphate (ADP). In the present thesis, this new whole blood, flow cytometry-based assay has been used to assess how infection and inflammation alter platelet numbers and function, and whether and how these platelet changes alter the innate immune response against these infections. The different studies involved both healthy volunteers as well as patients with various infectious diseases.

Studies in healthy volunteers

The level of platelet reactivity varies between individuals and is determined by both genetic and non-genetic factors (28). Studies have identified genetic variations which regulate platelet reactivity (29-31) or immune responses (32, 33) but have not interlinked these two processes. Although platelet reactivity is likely to influence inflammatory and immune responses and vice versa, the mechanisms of these interactions have not been well-established. In **chapter 2**, the interaction between platelets and inflammation was therefore explored in 500 healthy volunteers. In this study, we were also able to explore how the genetic variation that determine platelet reactivity can also exert its influence on the cytokine responses of whole blood and peripheral blood mononuclear cells (PBMCs).

As platelets are important in the development of thrombotic vascular events, platelet function inhibitors, especially aspirin and $P2Y_{12}$ receptor antagonists, have become the therapeutic and preventive cornerstones of cardiovascular diseases. Aspirin prevents prostanoid synthesis via inhibition of cyclooxygenase 1 (COX-1) and COX-2, thus abolishing platelet production of TxA2, an important platelet agonist (34). After the release of ADP from platelet dense granules, P2Y1 and $P2Y_{12}$ receptors are activated and act as an important feedback loop for other agonists including collagen, von Willebrand factor (vWF) and TxA2. Previous evidence has shown a clear role of $P2Y_{12}$ receptors in potentiating dense granule secretion, fibrinogen-receptor activation, thrombus formation and for irreversible platelet aggregation (12, 35, 36). $P2Y_{12}$ receptor antagonists such as clopidogrel, and the recently developed ticagrelor, have not only been shown to reduce platelet reactivity but also the formation of platelet-leukocyte complexes (37). In **chapter 3**, we explored the differential effects of platelets and platelet inhibition by ticagrelor on TLR2– and TLR4-mediated inflammatory responses. Through a series of *ex vivo* experiments, we determined the effects of isolated washed platelets on cytokine responses of peripheral blood mononuclear cells (PBMCs) from healthy volunteers to Pam3CSK4 and LPS stimulation. Additionally, in a double blind, placebo-controlled crossover trial of a single oral dosage of ticagrelor in healthy volunteers, we investigated cytokine production in blood exposed to Pam3CSK4 and LPS.

At present, more data on the effects of platelet inhibitors, including ticagrelor, on inflammation are available. Some studies for example, demonstrated the benefit of $P2Y_{12}$

inhibition in sepsis patients. Sepsis is a potentially lethal condition that arises when the body's response to an infection injures its own tissues and organs. The pathogenesis of sepsis involves a dysregulated host response with both exaggerated inflammation and immune suppression (38, 39). In sepsis, platelets can be activated due to the triggering of inflammatory and coagulation cascades, endothelial damage, and direct interactions with pathogens (40). Platelet activation may result in thrombocytopenia and microthrombosis, both of which have been associated with increased mortality in sepsis (41). A common *in vivo* model of systemic inflammation is the human endotoxemia model, whereby purified LPS (endotoxin) from *Escherichia coli* or other Gram-negative bacteria is administered intravenously to healthy volunteers. This model induces an acute systemic inflammatory response, which, although only partially, mimics the inflammatory response of early sepsis and other acute inflammatory conditions (42).
A frequent consequence of sepsis is the development of acute kidney injury (AKI) (43, 44). Platelets are thought to play a role in the pathogenesis of sepsis-associated AKI (45). In two phase II trials, bovine-derived alkaline phosphatase (AP) was shown to have a renal protective effect in critically ill patients with sepsis-associated acute kidney injury (AKI) (46). AP is a dephosphorylating glycoprotein enzyme with four different types of isoenzymes, namely placental, germ cell, intestinal, and tissue-nonspecific (liver/bone/kidney) AP. Three studies from the late 1980s showed that bovine-derived kidney AP inhibits platelet aggregation, although the precise mechanisms remained unclear (47-49). *In vitro* studies on renal cells showed that the anti-inflammatory effect of AP was related to its dephosphorylating activity on LPS and ADP (50). Recently, a human recombinant AP (recAP) was developed with enhanced stability and enzymatic activity (51), and in **chapter 4**, through a series of *ex vivo* experiments on whole blood from healthy volunteers and sepsis patients, we explored the platelet-inhibiting effect of recAP on platelets and involved pathways.
Concurrent with systemic inflammation in sepsis and critical illness, systemic hypoxia is common in critically ill patients, and is independently associated with adverse outcome. Tissue hypoxia may arise during inflammatory conditions as a result of enhanced metabolic demands, and decreased oxygen delivery (52). Hypoxia has also been implicated in the activation of coagulation, due to its association with thrombotic events (53). In rats, hypoxia has been shown to increase platelet reactivity (54). For the aforementioned reasons, it is plausible that hypoxia may contribute to altered platelet function and coagulopathy in critically ill patients, increasing their risks for organ dysfunction. However, the limited number of human studies available have mainly focused on the effects of hypoxia on plasmatic coagulation (55, 56), and human *in vivo* studies have only investigated the effects of hypoxia on platelet function during very mild (57) or using very short bouts of hypoxia (58). Although hypoxia and inflammation may both affect platelet function and plasmatic coagulation, human *in vivo* data on the

interaction between hypoxia and inflammation on coagulation are completely lacking. In **chapter 5**, we investigated the effects of systemic hypoxia during experimental human endotoxemia on platelet function, endothelial cell activation and plasmatic coagulation.

Studies in patients with infectious diseases

Changes in platelet function during infections may be important for two reasons: increased platelet reactivity may contribute to the development of thrombotic vascular complications, whereas platelet hyporeactivity may increase the risk for bleeding. The latter risk is especially high when thrombocytopenia is also present, like in haemorrhagic fevers, such as leptospirosis and dengue virus infections.

The risk for thrombotic vascular events, such as myocardial infarctions and stroke are indeed increased in infectious diseases (59). A high incidence of myocardial infarction and stroke was reported in patients with community acquired pneumonia (CAP) (60-62). *Streptococcus pneumoniae*, a Gram-positive bacterium, is a major cause of CAP. *S. pneumoniae* has been shown to interact with and activate platelets *ex vivo* (63), but *in vivo* studies of platelet function in animal models or patients are lacking. We speculated that excessive platelet activation may also contribute to the vascular comorbidity in pneumococcal infections. In **chapter 6,** we investigated platelet activation and hyperreactivity using a novel porcine model of intravenous *S. pneumoniae* infection. Furthermore, the utilization of bacterial whole genome sequencing for understanding pathogen virulence, including that of *S. pneumoniae*, allows prediction for the potential of a bacterial isolate to cause severe disease from the genome sequence alone (64, 65). We investigated therefore the association between the genotype of *S. pneumoniae* and disease outcome in 349 patients with pneumococcal bacteremia (**chapter 7).** More specifically, we explored the relevance of a phage-derived *pblB* gene, that encodes a platelet-binding protein of which the effects on platelet activation were previously unknown.

Acute bacterial infections are associated with an increased risk for CVE and stroke, but the magnitude of this risk depends on the type of infection (59, 66, 67). Clinical studies have shown a high incidence of thrombotic vascular complications in patients with Gram-positive bacterial infections (60, 68). This risk appears lower for urinary tract infections, which are usually caused by *Escherichia coli* or other Gram-negative bacteria (59, 69). In addition, infective endocarditis is predominantly caused by Gram-positive bacteria (70). Although studies have demonstrated both increased and decreased platelet activation in sepsis (71-74), its distinct pathogenic causes have not been considered. In **chapter 8**, we compared platelet reactivity in patients with Gram-positive or Gram-negative sepsis.

Another infection associated with increased platelet reactivity is HIV (75, 76). Platelets can internalize HIV, a process that is increased by platelet activation (77). On the other hand, increased platelet-monocyte complex formation and platelet reactivity to platelet

agonists are found in HIV-infected patients and this is thought to contribute to a higher risk for cardiovascular diseases (CVD) (75, 76). The impact of different regimens of antiretroviral therapy (ART) on platelet reactivity and PMC formation has not been rigorously studied. Intensification of ART with the integrase inhibitor raltegravir (RAL) was shown to suppress residual viral replication and reduce plasma levels of the coagulation marker d-dimer, although this finding was not replicated in another study (78, 79). A switch from a protease inhibitor to raltegravir decreased inflammatory and coagulation biomarkers (80). This suggests that a raltregravir-based regimen may also be associated with reduced platelet hyperreactivity In **chapter 9**, we studied platelet reactivity and platelet-monocyte aggregation in HIV-infected individuals receiving a raltegravir-based regimen, in comparison with those receiving nonnucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) regimens as well as healthy controls.

On the contrary, infections may also lead to decreased platelet reactivity. A study from our group demonstrated that dengue was associated with thrombocytopathy, which may contribute to the bleeding complications (81). Another infectious disease in which bleeding is an important complication is leptospirosis, which is caused by pathogenic spirochetes of the genus *Leptospira*. Leptospirosis incidence during outbreaks and in high exposure risk groups are estimated to exceed 100/100.000 per year. The clinical manifestations of leptospirosis range from a mild, self-limited febrile illness to a fulminant life-threatening illness with multi-organ failures (82). Hemorrhagic manifestations are clinically important complications occurring in up to 60% of all hospitalized patients, with severe pulmonary haemorrhage showing an alarmingly high mortality of >50% (83). The pathophysiological mechanisms responsible for bleeding in leptospirosis remains incompletely understood. We investigated platelet reactivity in patients with leptospirosis and its association with bleeding complications in **chapter 10**.

Outline of thesis

Studies in healthy volunteers

- Platelet number and reactivity are associated with immune responses and inflammation (**chapter 2**)
- Differential effects of platelets and platelet inhibition by ticagrelor on TLR2– and TLR4-mediated inflammatory responses (**chapter 3**)
- Human recombinant alkaline phosphatase inhibits *ex vivo* platelet activation in humans (**chapter 4**)
- Effects of hypoxia on platelet function and coagulation during systemic inflammation in humans *in vivo* (**chapter 5**)

Studies in patients with and animal model of infectious diseases

- Invasive pneumococcal disease leads to activation and hyperreactivity of platelets (**chapter 6**)
- Phage-derived protein induces increased platelet activation and is associated with mortality in patients with invasive pneumococcal disease (**chapter 7**)
- Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis (**chapter 8**)
- Reduced platelet hyperreactivity and platelet-monocyte aggregation in HIV-infected individuals receiving a raltegravir-based regimen (**chapter 9**)
- Thrombocytopathy contributes to bleeding complications in human leptospirosis (**chapter 10**)

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Chapter 2

Platelet number and reactivity are associated with immune responses and inflammation

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To be submitted.

Abstract

Introduction: Platelets are increasingly recognized as key cells in inflammation and immunity. Whether the number and hemostatic function of platelets are related with inflammation and the host immune responses and *vice versa* is still unknown. Platelet function and immune responses also have strong genetic determinants, but these have yet to be examined together.

Objectives: We systematically and comprehensively characterized the interaction between platelet function, immune responses, and their genetic variation in The 500-Human Functional Genomics (500FG) cohort of approximately 500 Caucasian, healthy individuals as part of the Human Functional Genomics Project (HFGP).

Methods: We analyzed the association of platelet number, platelet activation status and platelet reactivity to adenosine diphosphate (ADP) and collagen-related peptide (CRP-XL), with inflammatory markers and whole blood and peripheral blood mononuclear cell (PBMC) cytokine responses to stimulation with different Toll-like receptor ligands, bacterial and fungal stimuli. We then selected single nucleotide polymorphisms (SNPs) previously associated with platelet traits and related these to cytokine responses, and *vice versa.*

Results: Platelet numbers were strongly associated with plasma levels of IL-1β. Platelet activation and reactivity were positively correlated with *ex vivo* whole blood and PBMC IL-1β and IL-6 responses, whereas they were negatively correlated with IFN-γ responses. Addition of platelets to PBMC also upregulated IL-1β and IL-6 responses to LPS, *Candida albicans* and *Staphylococcus aureus* while it downregulated the IFN-γ responses. On the other hand, the addition of recombinant IL-6, IL-1β and IFN-γ did not alter platelet reactivity. Single nucleotide polymorphisms (SNPs) known to regulate platelet number, mean platelet volume and function correlated with cytokine responses to different pathogens.

Conclusion: In healthy individuals, platelet number is associated with plasma IL-1β levels. There are both functional associations as well as genetic overlap between platelet reactivity and cytokine responses. Our findings further show that platelets and their reactivity are interrelated with inflammation and immune responses.

Introduction

Platelets are traditionally known for their role in thrombosis and hemostasis. More recent evidence highlighted another role for platelets as important effector cells of inflammation and host defense (1, 2). Platelets are able to respond rapidly to threats by virtue of their large numbers and their ability to release different granules containing a diverse array of inflammatory cytokines, growth factors and inflammatory mediators (1, 3, 4). A multitude of platelet surface receptors and integrins mediate these hemostatic and inflammatory responses (1, 5, 6). Platelets are also able to interact with and change the phenotype of monocytes, lymphocytes and neutrophils (7, 8). Depending on the stimuli involved, platelets may enhance or dampen monocyte cytokine responses (9-11). Given the role of platelets at the cross-road of hemostasis, innate immunity and inflammation, platelet function is likely to influence inflammatory and immune responses and *vice versa*. The extent and direction of these interactions have not been well-established. To date, most data were derived from *in vitro* or animal studies [reviewed in (1, 2, 6)], and from clinical studies in patients with infections (12-14) or chronic inflammation (15-18). Whereas the level of platelet reactivity is consistent over time within an individual (19-22), it varies considerably between individuals and is determined by both genetic and non-genetic factors(19-26). The same applies to the capacity to produce cytokines (27). Studies have identified single nucleotide polymorphisms (SNPs) regulating platelet function (28-30) or immune responses (31, 32) but have not interlinked these two processes.

Using data from the 500 Functional Genomics (500FG) cohort, which is part of the Human Functional Genomics Project (HFGP; www.humanfunctionalgenomics.org), we take the first steps to systematically unravel the complex interactions between platelet function, inflammation and cytokine responses, and genetic variation.

In approximately 500 Caucasian healthy individuals, platelet function was assessed by whole blood flow cytometry as previously described (33). In short, P-selectin expression (a marker of platelet degranulation) and fibrinogen bound to the activated αIIbβ3 integrin (a marker for platelet aggregation) were measured after *ex vivo* stimulation of whole blood with adenosine diphosphate (ADP) and collagen-related peptide (CRP-XL), which is a glycoprotein VI (GPVI)-specific collagen mimetic. We selected these agonists due to their major role in platelet activation and representation of two distinct pathways: an early signaling of platelet activation through the GPVI receptor pathway and a later one through the $P2Y_1$ and $P2Y_{12}$ receptors (34, 35). Platelet count, plasma concentrations of the platelet α-granule protein β-thromboglobulin (β-TG), the plasmatic coagulation marker thrombin-antithrombin complex (TAT), and inflammatory cytokines and adipokines were also measured. Immune responses were assessed by measuring cytokine concentrations in whole blood and supernatant of

isolated peripheral blood mononuclear cells (PBMCs) to a range of synthetic, bacterial and fungal stimuli.

We first explored associations between platelet count, platelet reactivity, and TAT levels with a range of non-genetic host factors, inflammatory markers and cytokine responses. Significant correlations between platelets and cytokine responses were confirmed in separate laboratory experiments. Next, we investigated associations between known platelet SNPs with cytokine responses, and *vice versa*, between known cytokine SNPs with platelet reactivity. Finally, we examined the overlap of platelet SNPs with human-disease-associated SNPs.

Results

Characteristics of participants, platelet parameters and correlation with non-genetic host factors

Data from a total of 489 healthy participants from the 500FG cohort were analyzed for this study. Characteristics have been published recently (36). The median (IQR) age was 23 years (21-27 years) and 56% were female. The median BMI was 22.3 (20.7-24.4) and 83.1% had a BMI£25. Forty-eight percent of women used an oral contraceptive and 13.2% of subjects regularly used tobacco.

We obtained four measures of platelet reactivity by calculating the area under the curve (AUC) of P-selectin expression and platelet-bound fibrinogen in response to *ex vivo* stimulation with 8 increasing concentrations of ADP (P-selectin, APR, n=489; platelet-bound fibrinogen, AFR, n=396) or CRP-XL (CPR and CFR, both n=302). Representative platelet reactivity curves and data on the distribution of these platelet responses are shown in **Supplementary Fig. 1**. **Fig 1** illustrates the correlations between platelet count, platelet reactivity measures and plasma concentration of β-TG and TAT. As expected, the four readouts of platelet reactivity correlated well with each other, with the strongest correlations between those in response to the same platelet agonist. Whereas platelet reactivity readouts did not show an association with platelet count, there was a positive relation between β-TG plasma concentrations and platelets count, and this was reason to normalize β-TG levels for platelet mass in further analyses. The positive associations between total plasma concentration of β-TG and APR as well as TAT were no longer present after normalizing β-TG for platelet count.

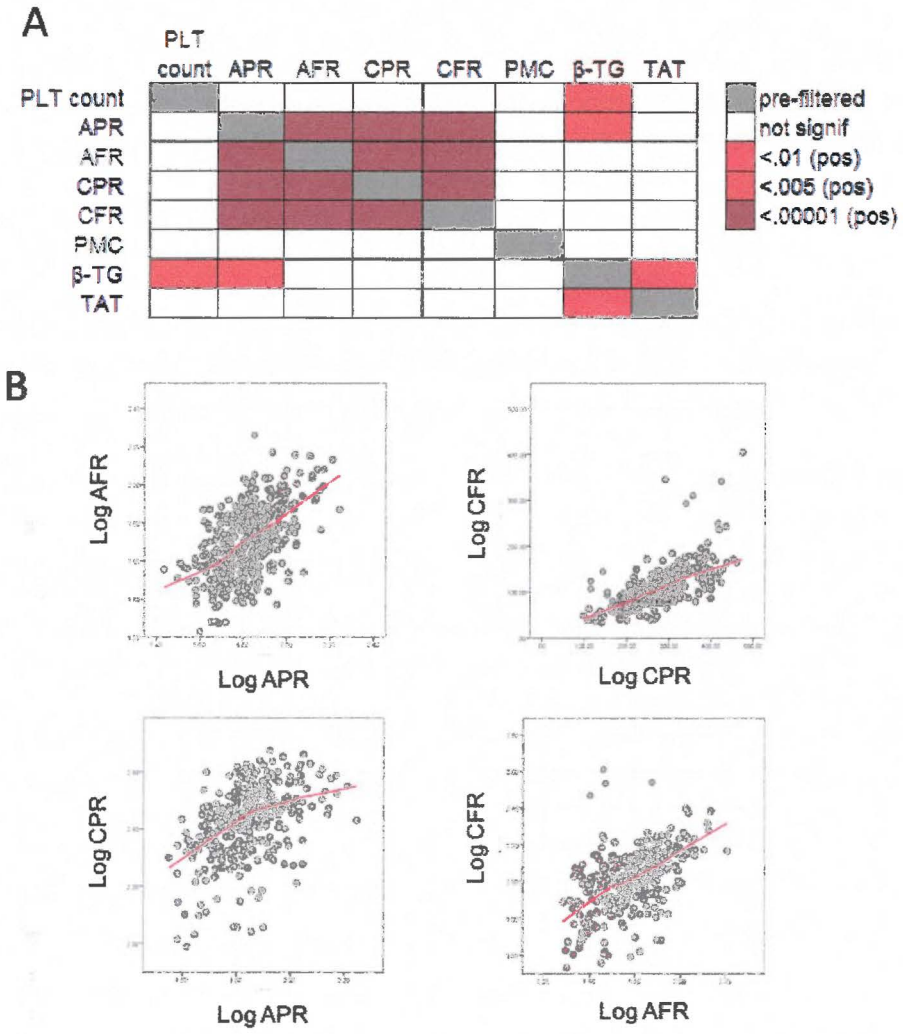

Figure 1. Correlations between platelet and plasmatic coagulation parameters.
(A) *P* values of the correlations of platelet and coagulation parameters, corrected for multiple testing using the False Discovery Rate (FDR) method. The color legend indicates the range of *P* values. (B) Scatterplots of highly significant correlations from (A). The red line shows the LOESS fit to the data. PLT count, platelet count; APR, ADP-induced P-selectin expression; AFR, ADP-induced platelet-fibrinogen binding; CPR, CRP-XL-induced P-selectin expression; CFR, CRP-XL-induced platelet-fibrinogen binding; β-TG, plasma concentration of β-thromboglobulin; TAT, plasma concentration of thrombin-antithrombin complex.

We next tested for associations of platelet parameters with age and a range of environmental and intrinsic non-genetic host factors. We did not find significant associations between platelet reactivity measures or β-TG levels with age, gender, BMI, sex, oral contraceptive usage, smoking and vitamin D concentrations. In contrast, females had a significantly higher platelet count than males with median (interquartile range, IQR) values of 280 (218-352) x 10^9/L vs. 251 (201-319) x 10^9/L (P=0.002), an observation previously reported by Biino et al (37). Oral contraceptive use is known to activate coagulation (38, 39) and females using oral contraceptives (n=155) indeed had higher TAT levels (3963 pM, 2526-5597 pM) compared with females not using oral contraceptives (n=166) (2824 pM, 1941-4215 pM; P<0.0001) and males (2640 pM, 1856-4235 pM; P<0.0001) in our study (**Supplementary Fig. 2**).

Associations of platelet and coagulation parameters with circulating cytokines, acute phase proteins and adipokines

We examined the interrelationship between platelet function and circulating concentrations of cytokines (IL-1β, IL-6, IL-1RA and VEGF-A), acute phase proteins (C-reactive protein, CRP, and alpha-1-antitrypsin, AAT) and adipokines (leptin and adiponectin) (**Fig 2**). We found a strong positive association between platelet count and the pro-inflammatory cytokine IL-1β, but not with IL-6, IL-1RA and VEGF-A (**Fig 2A**). Concentrations of these inflammatory cytokines also did not correlate with platelet reactivity and TAT levels. Both AAT and adiponectin levels were strongly positively correlated with β-TG/10^6 platelets, and to a lesser extent with platelet reactivity (**Fig 2B**). In contrast, AAT and adiponectin were negatively correlated with platelet count. Among the parameters measured, only TAT showed association with CRP level.

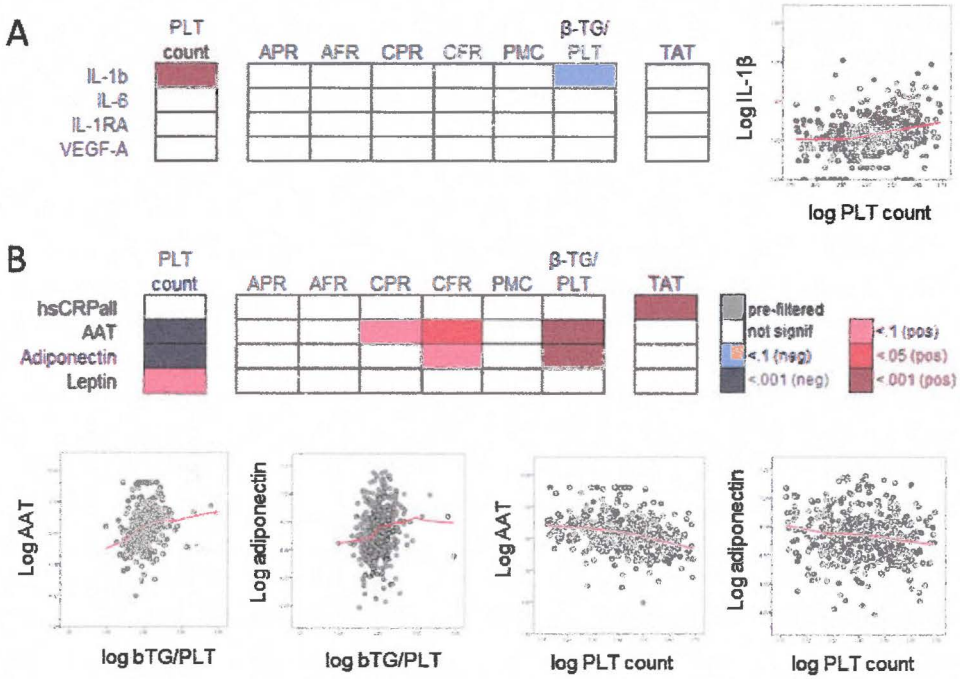

Figure 2. Correlations of circulating inflammatory mediators with platelet parameters and TAT.

(A) *P* values (FDR corrected) of correlations of circulating cytokines with platelet parameters and TAT as well as scatterplot of the correlation between IL-1β and platelet numbers. (B) *P* values (FDR corrected) of correlations of acute phase proteins (high sensitive C-reactive protein, hsCRP, and alpha-1-antitrypsin, AAT) and adipokines (leptin and adiponectin) with platelet parameters and TAT as well as scatterplot of the most significant correlations. The color legend indicates the range of *P* values. The red line shows the LOESS fit to the data. PLT count, platelet count; APR, ADP-induced P-selectin expression; AFR, ADP-induced platelet-fibrinogen binding; CPR, CRP-XL-induced P-selectin expression; CFR, CRP-XL-induced platelet-fibrinogen binding; β-TG/PLT, β-thromboglobulin/10^6 platelets; TAT, plasma concentration of thrombin-antithrombin complex.

Platelet reactivity is associated with cytokine responses

To capture the relationship between platelet reactivity and cytokine responses of immune cells, we measured and analyzed the production of the monocyte derived (IL-1β and IL-6) and lymphocyte-derived cytokines (IFN-γ) after *ex vivo* stimulation of whole blood and peripheral blood mononuclear cells (PBMCs) with the following five stimuli: two purified synthetic microbial ligands: LPS or Pam3CSK4; two bacterial stimuli: *Escherichia coli* and *Staphylococcus aureus*; and one fungal stimulus: *Candida albicans* conidia. This resulted in the analyses of a total of 21 cytokine measurements

for each of the participants in this study. We also tested the associations between the cytokine responses of PBMCs and platelet reactivity because previous experiments by our group revealed that isolated PBMC pellets still contain considerable numbers of platelets (data not shown).

In whole blood, we found an inverse association of platelet reactivity to CRP-XL and IFN-γ responses to *S. aureus* and LPS (**Fig 3A**). β-TG levels also showed and inverse association with the IFN-γ response to *S. aureus*, and TAT levels an inverse association with IFN-γ responses to LPS and *C. albicans*. In contrast, platelet reactivity to ADP was positively associated with IL-6 responses to *C. albicans* and *S. aureus* in whole blood and with LPS- and *E. coli*-induced IL-1β and IL-6 responses and Pam3CSK4- and *S. aureus*-induced IL-6 responses in PBMC isolates. In addition, β-TG showed a positive association with LPS-induced IL-6 and *E. coli*-induced IL-1β responses. Representative scatter plots are depicted in **Fig 3B-C.**

Next, we validated these observations of an opposite association of platelet reactivity with IFN-γ and IL-1β/IL-6 cytokine responses in a set of *in vitro* experiments. We incubated isolated PBMCs with LPS, *E. coli*, *C. albicans* and *S. aureus* in the presence and absence of isolated washed platelets. The need for direct cell to cell contact was assessed by using a transwell system. In line with the observations from our cohort, the addition of platelets decreased IFN-γ production in response to *C. albicans* and *S. aureus*, whereas IL-1β and IL-6 responses to all stimuli were significantly increased (**Fig 3D**). Physical separation of PBMC and platelets reversed these cytokine-modulating effects of platelets.

We also investigated whether direct effects of cytokines on platelet function contributed to the observed interrelationship of cytokine responses with platelet reactivity in our cohort. Platelet-rich plasma (PRP) was incubated with recombinant IL-1β, IL-6 or IFN-γ and the expression of P-selectin and fibrinogen binding were measured. These cytokines did not have any effect on P-selectin expression and platelet-fibrinogen binding with or without additional ADP and CRP-XL stimulation (**Supplemental Fig 3**). The recombinant cytokines also did not lead to differences in PMC formation (data not shown).

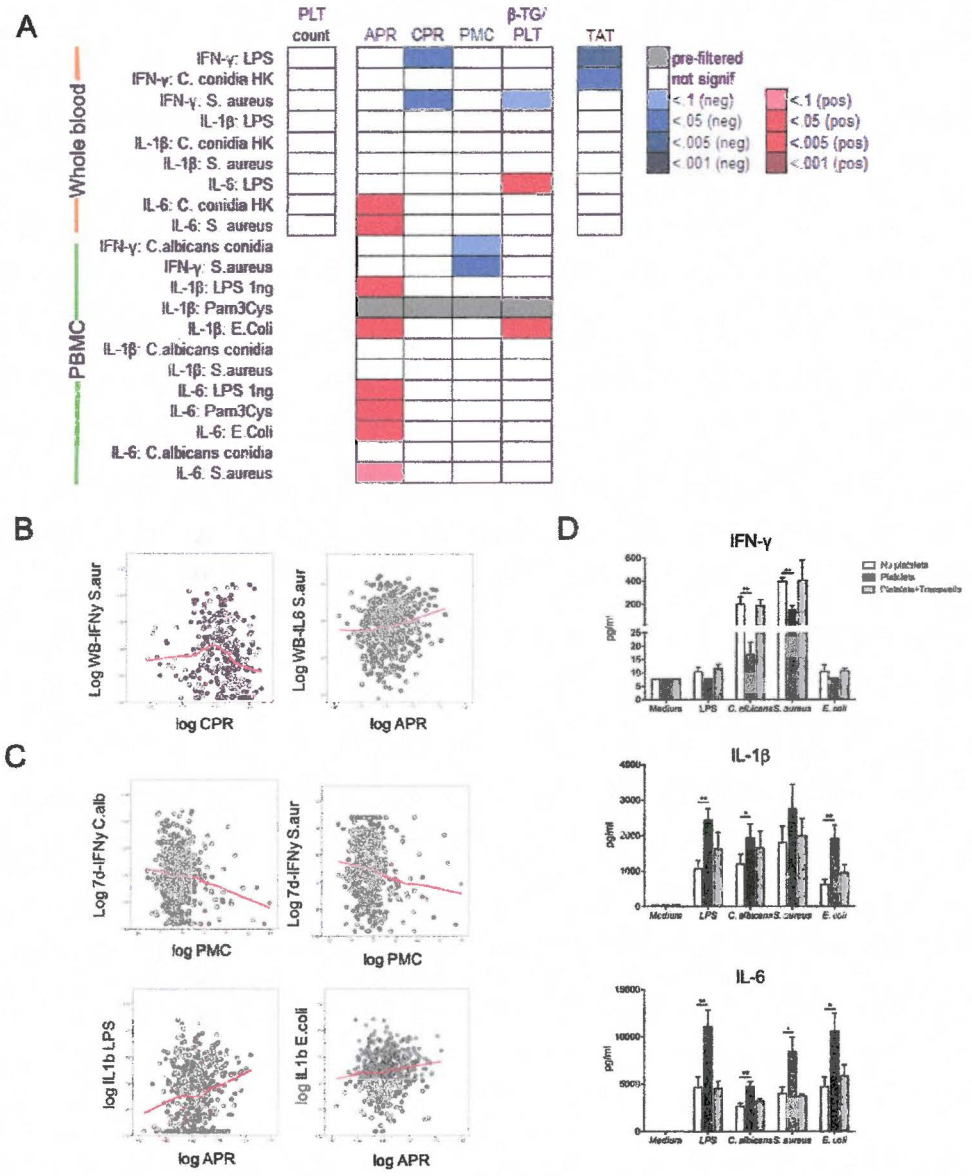

Figure 3. Associations of platelets with cytokine responses.

(A) *P* values (FDR corrected) of the correlations of peripheral blood mononuclear cells (PBMCs) and whole blood cytokine responses with platelet parameters and TAT. The color legend indicates the range of *P* values. (B) Scatterplots of the highly significant correlations of whole blood cytokine responses from (A). (C) Scatterplots of the highly significant correlations of PBMC cytokine responses from (A). The red line shows the LOESS fit to the data. (D) PBMCs were co-cultured for 24 h with isolated washed platelets and Pam3CSK4 or LPS with or without physical separation of platelets and PBMC using a Transwell system. Interleukin (IL-)1- β, IL-6 and IFN-γ concentrations were determined in the culture supernatant. Platelets were added in a ratio of 150 platelets for every PBMC. Presented data are means with standard error of the mean (SEM) from 6 healthy donors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Genetic relationship with platelet parameters

There is a marked and reproducible variation in platelet number and reactivity between individuals and genetic association studies have identified an ever growing number of SNPs that modify these platelet parameters (30, 40, 41). We aimed to associate genetic variations that determine platelet parameters with cytokine responses and *vice versa*. Genotyping in our cohort was performed using Illumina HumanOmniExpressExome SNP chip and was imputed to obtain genotypes at ~7 million SNPs. We first tested association of ~4 million SNPs, showing a minor allele frequency ≥5%, with platelet count, platelet reactivity to ADP and CRP-XL (APR, AFR, CPR, CFR), PMC formation, plasma β-TG and TAT complexes. No significant genome-wide associations for any of these parameters were found with the exception of one novel significant quantitative trait loci (QTL) for TAT complexes (rs1149961; $P < 5 \times 10^{-8}$; **Fig 4A**). Next, we examined the associations of SNPs that were identified in previous studies with platelet traits in our cohort. A list of 133 SNPs was made, including those implicated in platelet number (29, 42-45), platelet reactivity (23, 28, 30, 46-48) and mean platelet volume (MPV) (29, 42, 49). Even though the latter parameter was not measured in our cohort, it was included as platelet size is related to the number of integrins on the platelet membrane (23, 42) and genetic variance in MPV was also associated with platelet reactivity (29).

Seventy-four SNPs were detected in our cohort and 30 (41%) of these showed at least one suggestive association with platelet reactivity, platelet number PMC formation or β-TG (**Fig 4B**). We not only replicated a number of prior associations, but also identified a variety of novel associations. The strongest associations with platelet reactivity were with SNPs in the genes encoding the surface proteins glycoprotein VI (collagen receptor; *GP6*), ADP receptor P2Y1 (*P2RY1*), platelet endothelial aggregation receptor 1 (*PEAR1*) and in the gene *JMJD1C*. As expected, rs1613662 and rs1671152 in *GP6* were positively associated with CRP-XL-induced reactivity, while rs3755711 and rs701265 in *P2RY1* inversely with ADP-induced reactivity. In contrast, significant associations with reactivity to both ADP and CRP-XL were found for rs11264579 in *PEAR1* and rs10761741 and rs2393967 in *JMJD1C*, supporting previous data (30) that these loci function in a relatively agonist-independent manner. *PEAR1* is involved in the amplification of αIIbβ3 activation in a PI3K-dependent manner (50) whereas *JMJD1C* encodes a histone demethylase and plays a role in megakaryocyte development and function (51, 52).

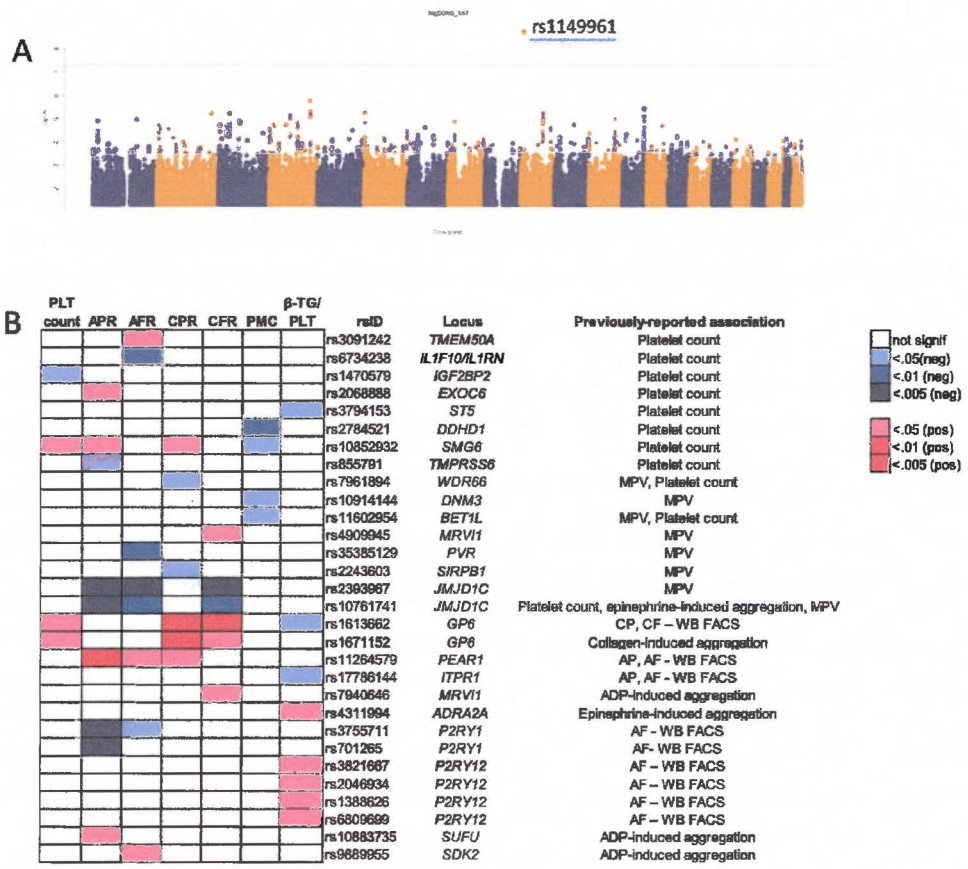

Figure 4. Manhattan plot showing the genome-wide QTL mapping result and associations between platelet SNPs and platelet parameters.
(A) Manhattan plot showing the mapping result for thrombin-antithrombin complex (TAT). (B) The color legend indicates the range of *P* values. *P* values were obtained from linear regression model of platelet parameters on genotype data. MPV, mean platelet volume; CP, collagen-induced P-selectin expression; CF, collagen-induced platelet-fibrinogen binding; AP, ADP-induced P-selectin expression; AF, ADP-induced platelet-fibrinogen binding; WB FACS, whole blood flow cytometry.

Genetic relationship between platelet parameter and cytokine responses

Next, we examined whether these 30 platelet SNPs in our cohort were associated with whole blood and PBMC cytokine responses. Twenty-two SNPs showed at least marginal association ($p<0.05$) with one or more cytokine responses and five (rs10761741, rs6734238, rs3794153, rs3091242 and rs11602954) had more than five associations (**Fig 5A**). SNPs in the genes GP6 and *P2YR1*, which had strong associations with reactivity to CRP-XL and ADP, respectively, were not associated with cytokine responses with

the exception of a marginal association of rs3755711 in *P2YR1* with IL-17 responses to *Mycobacterium tuberculosis*. In contrast, rs10761741 in *JMJD1C* showed a variety of associations with PBMC and whole blood cytokine responses. In PBMC stimulations, the minor allele in this SNP was negatively associated with IFN-γ (*to Borrelia burgdorferi and Bacteroides*) and IL-22 (*to B. Burgdorferi, Bacteroides and M. tuberculosis*) responses, and positively associated with IL-6 (to CpG and polyIC) and TNF-α (to *S. aureus* and Pam3CSK4) responses. In whole blood stimulations, negative associations were found for IFN-γ, IL1-b and TNF-α responses to *Candida*. In contrast, rs2393967 in *JMJD1C* and rs11264579 in *PEAR1*, which were also relatively strongly associated with platelet reactivity, only showed marginal association with TNF-α responses to Pam3CSK4, *S. aureus* (both PBMC) and *Candida* (whole blood, rs2393967) and IL-6 to *S. aureus* and IFN-γ to *Cryptococcus* (rs11264579).

Of the other four SNPs with more moderate platelet effects, but with more than five cytokine associations, the strongest associations with cytokine responses was found for rs6734238 (*IL1F10/IL1RN*) region. This SNP is located downstream of *IL1F10* and upstream of *IL1RN* (53), an inflammatory gene region of the interleukin-1 cytokine gene family (54), and had been associated with C-reactive protein (CRP) levels (55, 56). Recently, this variant was reported to be associated with mean corpuscular hemoglobin and leukocyte and platelet number (29). In our cohort, rs6734238 was associated with plasma β-TG levels, but also with reduced IFN-γ, IL-17, IL-22, IL-1β, IL-6 and TNF-α responses to the different ligands in PBMC and whole blood. The *ST5* variant rs3794153, which had previously been associated with platelet count (29), was associated with APR in our cohort and had overlapping multiple positive associations with IL-1β, TNF-α and IFN-γ responses. ST5 regulates the activation of MAPK1/ERK2 (57), which is known to play important role in both immune responses (58) and platelet activation (59). rs3091242 in *TMEM50A* has previously been related with platelet number (29) and was associated in our cohort with APR. This SNP was associated with IFN-g/IL-22 and IL1-β/TNF-α which were largely in the opposite direction. Finally, rs11602954 in *BET1L* was positvely associated with multiple cytokine responses.

Recently, we reported different SNPs related with cytokine responses in our cohort (60). We therefore tested whether these cytokine SNPs were also associated with platelet responses in our cohort (**Fig 5B**). Multiple associations with platelet traits were found. Notably, rs10908219, located near *FGF19*, *FGF4*, and *FGF3*, was positively associated with ADP and CRP-XL-induced αIIbβ3 integrin activation whereas rs4491463 was negatively associated with ADP-induced αIIbβ3 integrin activation and levels of β-TG. We also found several other cytokine SNPs associated with a single platelet trait. rs4496335 near IL1RN had previously been associated with IL-1RA levels (61), while rs6834581, rs10108108, rs10908219, rs4491463 and rs7256586 were shown to be associated with a cytokine responses to a range of different pathogens (60).

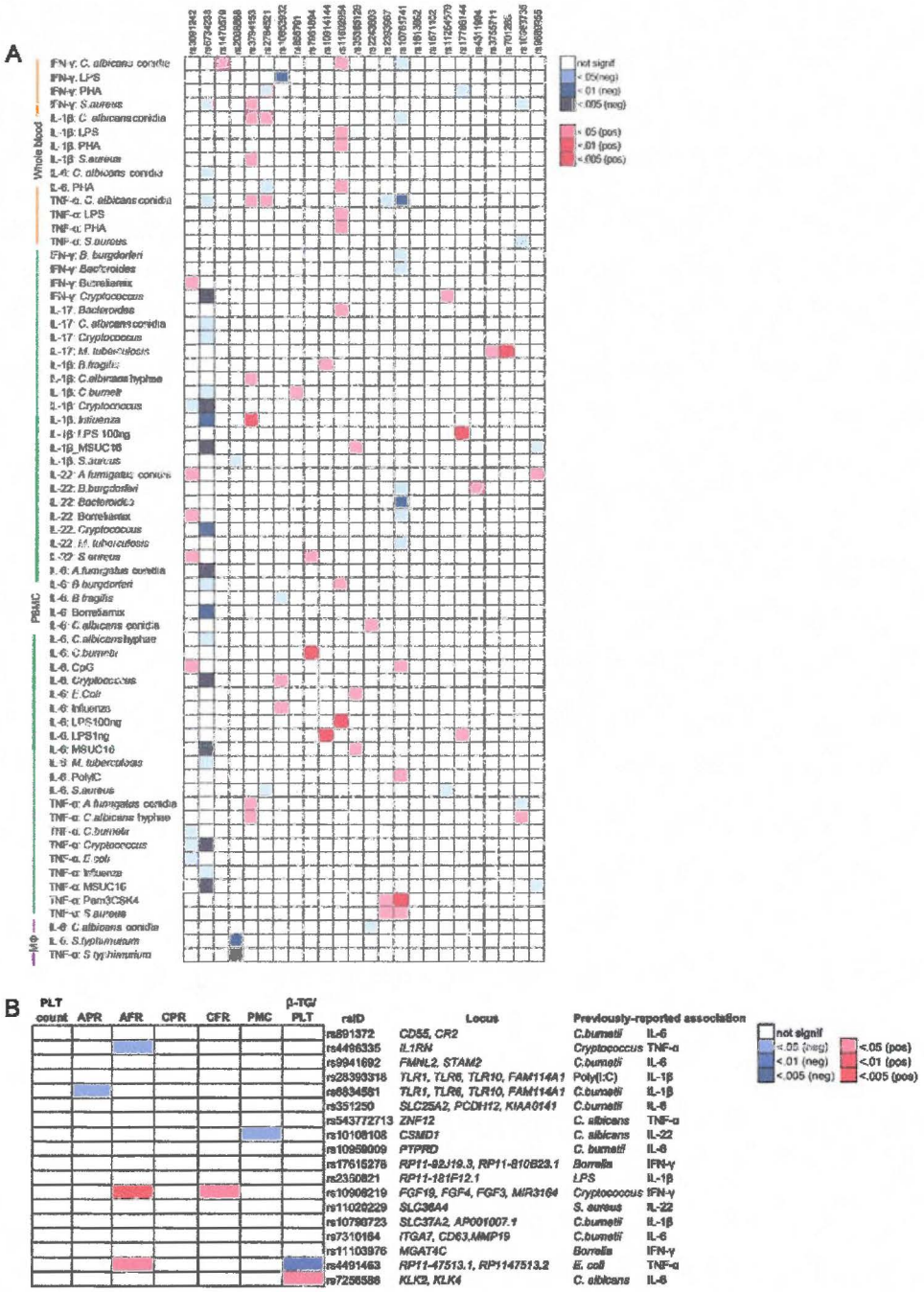

Figure 5. SNPs associated with overlapping platelet traits and cytokine responses in whole blood and PBMCs.

(A) The *P* values of the associations of cytokine responses with platelet SNPs and *P* values were from linear regression analysis of cytokine responses on genotype data. (B) The *P* values of the associations of platelet parameters with cytokine SNPs. *P* values were from linear regression analysis of platelet parameters on genotype data. The color legend indicates the range of *P* values.

Platelet SNPs overlap with SNPs associated with immune-mediated diseases

We tested whether SNPs that were previously associated with human diseases, particularly with immune-mediated diseases, are enriched with platelet SNPs. We extracted GWAS SNPs from the National Human Genome Research Institute GWAS catalog (62) and binned them into eight categories on the basis of their association with different human phenotypes (Online Methods). Next we identified all SNPs that were associated with platelet levels at P < 1x10-4 and tested whether they are also GWAS SNPs or their proxies (r2 >0.8). We found that 0.15% of immune-mediated disease–associated SNPs were also associated with platelet traits (**Fig 6**).

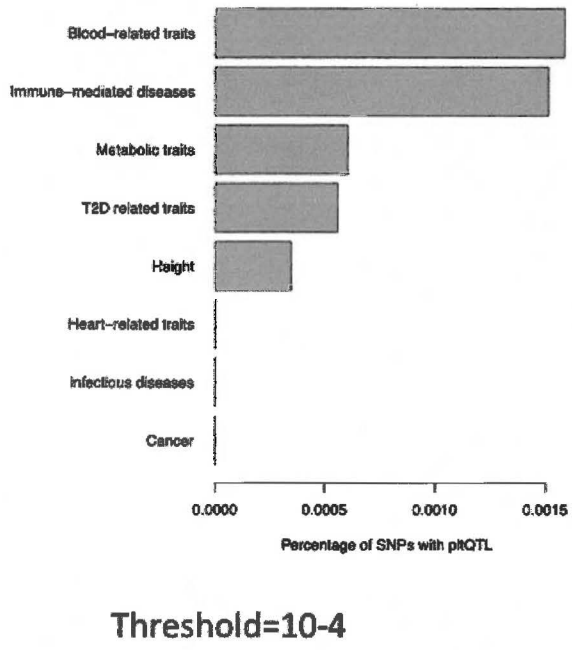

Figure 6. Platelet SNPs overlap with SNPs associated with immune-mediated diseases. The percentage of SNPs associated with each category of disease that also qualified as platelet SNPs.

Discussion

In the present study, we explored the interactions between platelet function, inflammation, cytokine responses, and genetic variation by assessing these elements together in a cohort of approximately 500 healthy individuals. We found that platelet count was strongly associated with IL-1β concentrations, but not with other cytokines. Platelet reactivity correlated inversely with *ex vivo* IFN-γ responses, a T cell-derived cytokine, and positively with IL-1β/IL-6 responses, which are monocyte-derived cytokines. We were able to validate these opposite associations in a set of *ex vivo* experiments, whereby platelets attenuated IFN-γ responses to *C. albicans* and *S. aureus*, and increased IL-1β/IL-6 responses to the aforementioned stimuli, *E. coli* and LPS. The effects of platelets on cytokine responses were dependent on direct contact between platelets and PBMCs. On the other hand, recombinant IFN-γ, IL-1β and IL-6 did not alter the platelet expression of P-selectin or activation of the aIIbb3 integrin. Consistent with these findings were genetic associations of variants in genes related with platelet function and cytokine responses. There was a substantial overlap of the associations between previously-reported platelet SNPs with both platelet traits and cytokine responses. We also found associations between cytokine SNPs and platelet traits, and that platelet SNPs intersect with SNPs of immune-mediated diseases.

Platelet count was strongly associated with circulating IL-1β levels, but not with other cytokines, suggesting that platelets either contributed significantly to the amount of circulating IL-1β or that IL-1β drives platelet production. IL-1β is an inflammatory cytokine which is mainly produced by monocytes, macrophages, and dendritic cells (63). Platelets were known to harbor and release mature IL-1β upon activation (64, 65), but their contribution to circulating IL-1β levels is unknown (66). Megakaryocytes also respond to IL-1β by increasing platelet production (67-69) through the increase in thrombopoietin and transcription factors (70). They were also shown to secrete IL-1β as a positive feedback loop for megakaryopoiesis (71). Our results further show that *in vivo*, platelet numbers and plasma IL-1β are interrelated even in a non-inflammatory, non-infectious setting.

Previous *ex vivo* laboratory studies have determined the effects of platelets on cytokine responses (9, 11, 72-74). This present study adds by further showing, in our cohort of healthy individuals, the *in vivo* differential associations of platelet reactivity with IFN-γ and IL-1β/IL-6 responses to various synthetic and bacterial ligands. To the best of our knowledge, the opposing effects of platelets on the monocyte-derived and T-cell-derived cytokines have not been reported previously. There are several possible mechanisms that can explain the associations between platelet reactivity and cytokine responses. First, more reactive platelets may interact more strongly with PBMCs in our *ex vivo* system. Platelets are present in isolated PBMCs preparation and their complete elimination is not

possible (9). Both IL-6 and IL-1β are cytokines synthesized in a nuclear factor-kappa B (NF-kB)–dependent pathway (75-77). The interaction between P-selectin and monocyte P-selectin glycoprotein ligand (PSGL)-1 triggers nuclear translocation of NF-kB and activation of the NF-kB-dependent inflammatory genes (72, 73), and this mechanism likely contributes to the increased *in vitro* IL-1β/IL-6 responses of PBMCs with the addition of platelets, as also previously found by our group (9). The second mechanism may be that more reactive platelets secrete more immune-modulating proteins. Platelet microparticles could prevent the differentiation of peripheral blood–derived regulatory T cells into IL-17– and IFN-γ–producing cells, and as such inhibit IL-17 and IFN-γ production (78). Activated platelets may also secrete IL-1β generated from intraplatelet pre-mRNA that undergoes post-transcriptional splicing and translation upon activation (64, 65), which may in turn modulate inflammatory response in leukocytes (65). IFN-γ inhibits IL-1β at the level of transcription (79) and suppresses IL-1 production and infection-induced immunopathology through the inhibition of NLRP3 inflammasome (80), both in a nitric oxide (NO)-dependent manner. IFN-γ may also attenuate the binding of NF-kB to the IL-1β promoter independently of NO (81). The observed opposite modulatory effect on cytokine responses by platelets in our study may be a result of this counter-regulatory activity. Our *in vitro* findings that recombinant cytokines failed to induce changes in platelet reactivity were similar to that of Brown et al., whereby IL-1β pre-treatment did not potentiate platelet reactivity to ADP and CRP-XL (66). IL-1β, similar to LPS, is known to be an atypical agonist that failed to promote platelet degranulation that increases surface P-selectin expression or platelet-fibrinogen binding (66, 82).

The third mechanism that may explain the association between platelet reactivity and cytokine response is the genetic overlap between these functions. Indeed, we found substantial overlap of our platelet SNPs with cytokine responses, indicating a shared genetic influence on regulatory and functional mechanisms among the different cells. Previously, platelet SNPs were already found to overlap with erythrocytes, leukocyte and lipid traits (29). We found that SNPs in the platelet surface receptors *GP6*, *P2Y12* and *PEAR1* that were strongly associated with platelet reactivity, displayed no or only weak overlapping associations with cytokine responses. In contrast, *JMJD1C* showed strong associations with platelet reactivity and a range of associations with PBMC and whole blood cytokine responses, which may be due to its effect on gene expression. *JMJD1C* is involved in epigenetic regulation, probably through histone demethylation (83), and in androgen receptor-mediated transcription (84). This gene plays a role in megakaryocytes proliferation (51) and has also been associated with leukocyte (85) and platelet count, MPV and platelet reactivity (28), indicating its multilineage effects on hematopoiesis. To our knowledge, variations in these aforementioned genes have previously not been related to immune function.

Other SNPs with more modest associations with platelet function overlapped with broader cytokine responses, indicating their more dominant role in the inflammatory pathways as these SNPs were located in genes which were known to regulate immune properties or pathway: the *IL1F10/IL1RN* region, *ST5* and *TMEM50*. The *IL1F10/IL1RN* region for example, had been associated with several inflammation-related biomarkers, including CRP, IL-1RA, and fibrinogen (86-88). *IL1F10* encodes IL-38 which inhibits Th17 immune responses and stimulates IL-6 cytokine production in vitro (89). *IL1RN* encodes IL-1 receptor antagonist (IL-1RA), which regulates diverse interleukin-1-related immune and inflammatory responses, as it prevents receptor binding of either IL-1α or IL-1β (63). *ST5* encodes for the protein activating MAPK1/ERK2 (57), which regulates cytokine production via both transcriptional and post-transcriptional mechanisms (90). Interestingly, the *TMEM50A* variant was also associated with opposite directions of IFN-γ/IL-22 and IL-1β/TNF-α responses, which mirrored the functional associations between platelet reactivity and opposite IFN-γ and IL-1β/IL-6 responses. *TMEM50A*, located at the *RH* gene locus, is a gene of unknown function and was described as showing homology to an open reading frame on chromosome 21, which is an IFN/IL-10 receptor gene cluster (91, 92). In support of our findings that platelet function and cytokine responses overlap each other at the functional and genetics level, we found that approximately 0.15% of immune-mediated disease-associated SNPs were also platelet SNPs. It is plausible that SNPs with overlapping traits are pleiotropic genetic variants, in which the single gene or genetic variant have independent effects on platelets and the inflammatory response. Locus level views of overlapping trait scans already suggested extensive pleiotropy in some platelet-related regions such as *ABO, SH2B3, HBS1L* (41). The limitations in this study include the unavailability of MPV as an outcome measure and that CRP-XL-induced platelet reactivity was only measured in 302 individuals. Furthermore, our conclusions on the interaction between platelets and cytokine responses are derived from separate measurements; *in vivo* platelet reactivity and platelet counts were assessed once blood was drawn whereas cytokine responses were measured after 24h, 48h and 7 days of incubation with various stimuli. This study did not examine the associations of SNPs from other studies which are rare frequency allele and were not present in this cohort. Lastly, our study was unable to demonstrate whether the SNPs with overlapping platelet and cytokine associations were pleiotropic variants.

In conclusion, we found that platelet counts are associated with plasma IL-1β levels. We also showed both functional associations as well as genetic overlap between platelet reactivity and cytokine responses. The overlap of SNPs regulating platelet traits and cytokine responses further shows that platelets and their reactivity are interrelated with the inflammatory and immune response. Additionally, pleiotropic SNPs may provide important clues to the new pathways and functions of genes in inflammation beyond their recognized role in shaping the platelet traits. Future studies would be required to

address the functional and genetic mechanisms by which circulating IL-1β is associated with platelet numbers, as well as novel pathways regulating inflammation and platelet function.

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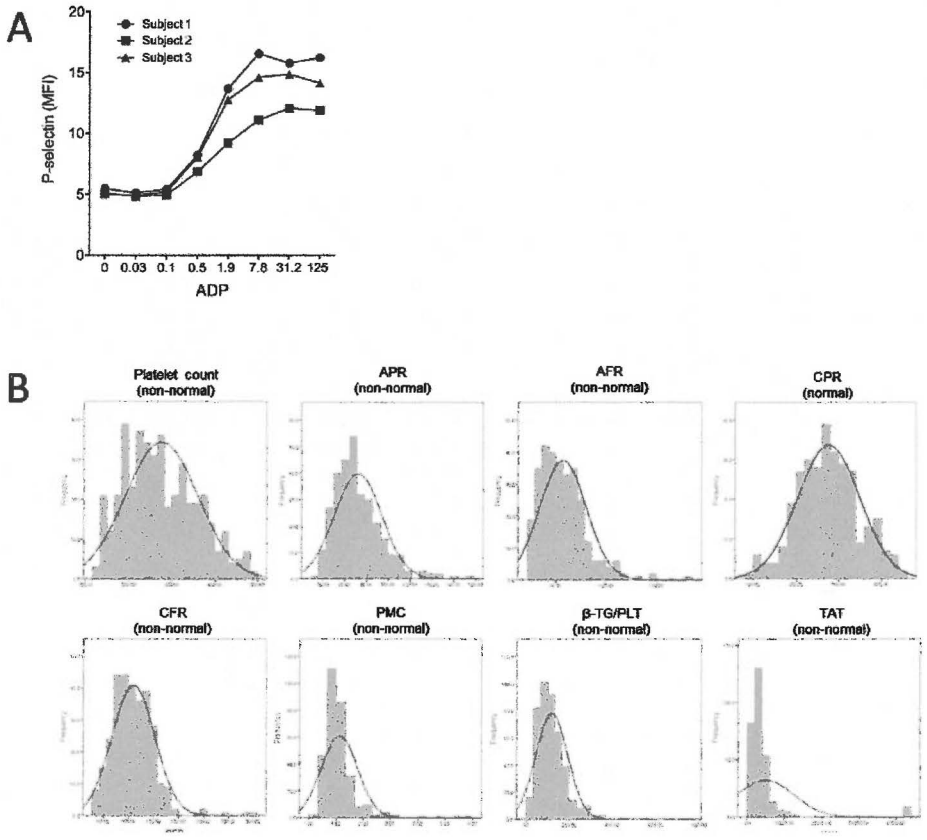
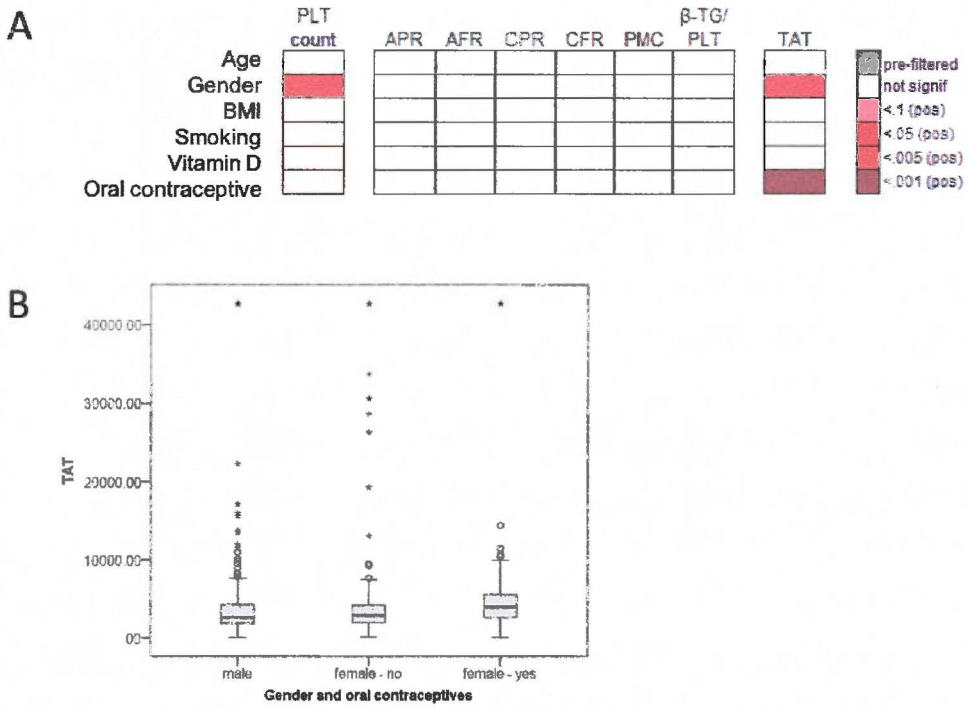
Supplemental Figures

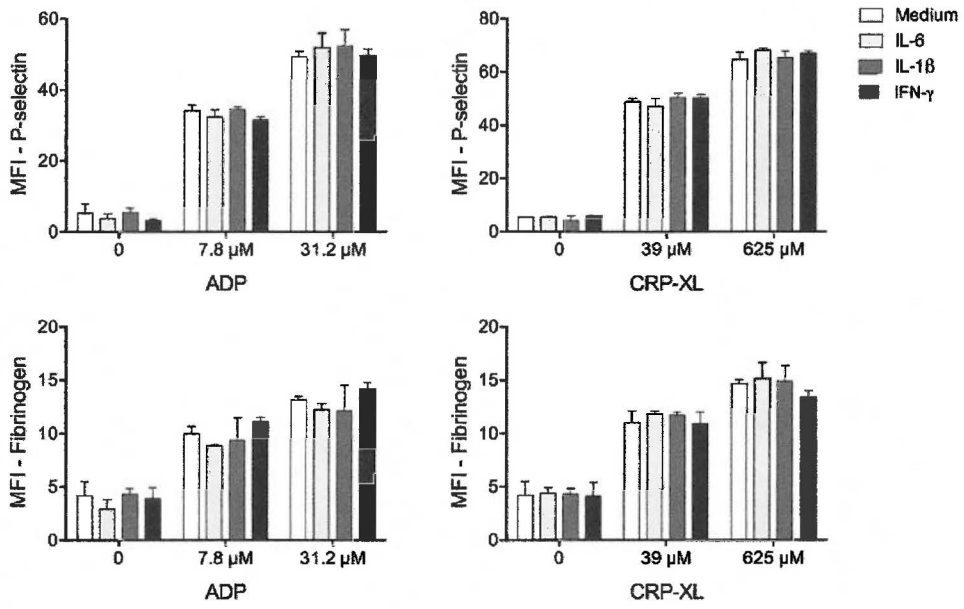


Supplemental Figure 1. Representative plot of the platelet reactivity curve and histograms of the platelet and coagulation parameters.

(A) Sample curves of ADP-induced P-selectin expression on platelets, as measured by whole blood flow cytometry and depicted as median fluorescence intensity (MFI). (B) Histograms showing distributions of the platelet and coagulation parameters. All values of the platelet and coagulation parameters were log10 transformed. Normality of the distribution was tested using Shapiro-Wilk test for normality with a cut-off of $P<0.05$.



Supplemental Figure 2.
(A) The *P* values (FDR corrected) of the correlations between platelet and coagulation parameters with age, BMI, sex, oral contraceptive usage, smoking and vitamin D concentrations. The color legend indicates the range of *P* values. (B) Comparisons of plasma levels of thrombin-antithrombin complex in subjects with and without oral contraceptive use.



Supplemental Figure 3. Platelet activation and reactivity after stimulation with ADP or CRP-XL were not affected by recombinant IL-1β, IL-6 and IFN-γ.

Platelet-rich plasma (PRP) from healthy donors was pre-treated with medium, recombinant IL-1β, IL-6 and IFN-γ (100 ng/ml) for 60 minutes in 37°C and subsequently stimulated with the platelet agonists adenosine diphosphate (ADP; 7.8 and 31 µM) and collagen-related peptide (CRP-XL; 39 and 625 µM). The median fluorescence intensity (MFI) of the platelet surface expression of P-selectin and platelet-fibrinogen binding was measured by using flow cytometry. Data are presented as means with standard error of the means (SEM) from 6 healthy donors.

Methods (online supplement)

Study population

Ethics statement

The 500FG study was approved by the Ethical Committee of Radboud University Nijmegen (NL42561.091.12, 2012/550). Inclusion of volunteers and experiments were conducted according to the principles expressed in the Declaration of Helsinki. Samples of venous blood were drawn after written informed consent was obtained.

Cohort description

The 500 Functional Genomics (500FG) study was performed in a cohort of 534 healthy individuals of Caucasian origin and is part of the Human Functional Genomics Project (HFGP). The volunteers were sampled between July 2013 and December 2014 at the Radboud university medical center. The Netherlands. Inclusion criteria were age>18 years and Western-European descent. Exclusion criteria were pregnancy/breastfeeding, chronic or acute disease at the time of sampling, and use of any medication in the last month before the study.

Measurements of platelet function and coagulation activation

Platelet reactivity

Venous blood was collected in citrated Vacutainer tubes (3.2% sodium citrate; Becton Dickinson, USA). Platelet reactivity was determined by a whole blood flow cytometry assay that had been described earlier (33, 93). In short, the platelet membrane expression of the alpha-granule protein P-selectin and the binding of fibrinogen to the activated integrin αIIbβ3 are measured in unstimulated samples and after *ex vivo* platelet stimulation by 8 increasing concentrations of adenosine diphosphate (ADP, 7.8-125 μM, Sigma-Aldrich, USA) or crosslinked collagen-related peptide (CRP-XL, 9-625 ng/ml, kind gift from Prof. dr. R. Farndale, Cambridge, UK). Whole blood was added to a mixture of HEPES-buffered saline and saturating concentrations of PE-labeled anti-CD62P (P-selectin; Bio-Legend, San Diego, USA), FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK) and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). After 20 minutes incubation at room temperature, 0.2% paraformaldehyde was added and samples were analyzed using a FC500 flow cytometer within 4 hours (Beckman Coulter, France). Platelets were gated based on their forward- and sideward-scatter properties and positivity for CD61, which was

defined as a median fluorescence intensity (MFI) exceeding the that of the matched isotype control; 10 000 single platelets were measured in each sample. Next, ADP- and CRP-XL-induced platelet P-selectin (termed as APR and CPR) and platelet-fibrinogen reactivity to either ADP or CRP-XL (termed as AFR and CFR) were determined by calculating the area under curve from the MFI of CD62P or fibrinogen on CD61-positive events generated from the 8 increasing concentrations of ADP or CRP-XL.

Platelet-monocyte complexes
The formation of PMC, which is considered a sensitive marker for platelet activation (94) was determined by incubating citrated whole blood with PC7-labelled anti-CD61 and PE-labelled anti-CD14 [a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein; Bio-Legend] as a monocyte identification marker. Optilyse B (Beckman Coulter, USA) was added after 30 min followed by distilled water. The PMC formation was quantified based on the MFI of CD61 on CD14-positive cells.

β-thromboglobulin and thrombin-antithrombin complexes
Plasma concentrations of the platelet α-granule protein, β-thromboglobulin (β-TG), a plasma soluble marker for platelet activation, and thrombin-antithrombin (TAT) complexes, a marker for coagulation activation, from all samples were measured at once using ELISA as previously described (95). Human β-thromboglobulin (MAB393, BAF393) was purchased form R&D systems, Abbington, UK, and sheep anti-human thrombin (SAHT-AP, SAHT-HRP) antibodies was purchased from Kordia/Affinity Biologicals, USA.

Stimulation of whole blood and peripheral blood mononuclear cells

Whole blood experiments
100µl of heparin blood was added to a 48 wells plate and subsequently stimulated in a total volume of 500µl for 48 hours at 37°C and 5% CO_2. Supernatants were collected and stored at -20°C until used for ELISA.

Peripheral blood mononuclear cell (PBMC) experiments
PBMC were isolated by density centrifugation of diluted EDTA-anticoagulated blood over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline and suspended in medium (RPMI 1640) supplemented with gentamicin (10 mg/mL), L-glutamine (10 mM) and pyruvate (10 mM). The PBMC were counted in a Coulter counter (Coulter Electronics, Buckinghamshire, England) and their concentration was adjusted to $5x10^6$/mL. 5×10^5 PBMC in a total volume of 200 µL

per well were stimulated in round-bottom 96-wells plates (Greiner) with the different stimuli for either 24 hours or 7 days at 37°C and 5% CO_2. When cells were stimulated for 7 days this was done in the presence of 10% human pooled serum. Supernatants were collected and stored in -20°C until used for ELISA.

ELISA analysis

Samples of all experiments were measured at once using the following ELISA kits:

In the 24-hour PBMC stimulation experiments we measured concentrations of human IL-1β, IL-6 as well as TNF-α (PeliKine Compact or R&D Systems). Supernatants of the 7 days stimulation assays were used to measure IL-22, IL-17 or IFN-γ (PeliKine Compact or R&D Systems). For the whole blood samples IL-6, TNF-α, IL-1β as well as IFNγ levels were determined (PeliKine used for IL-6 and TNF-α measurements (PeliKine Compact or R&D Systems).

Measurements of circulating mediators and vitamin D

The circulating mediators hsCRP, leptin, adiponectin, and alpha-1 antitrypsin (AAT) were measured in EDTA plasma using the R&D Systems DuoSet ELISA kits following the manufactures standard protocol. The plasma cytokines IL1Ra were measured using R&D Quantikine kits following the manufacturer's standard protocol. Additional details are available in the Supplemental Information. 25-hydroxy vitamin D3 was analyzed by LCMSMS after protein precipitation and solid-phase extraction. Additional details are available in the Supplemental Information.

Correlation analysis

Associations for each measurements of platelet count, platelet function and TAT in connection with the acute reactive proteins, adipokines, circulating cytokines and whole blood/PBMC cytokine responses were analyzed using Spearman correlation coefficient in combination with Benjamini-Hochberg correction to account for multiple hypothesis testing (significance threshold $\alpha \leq 0.1$). For whole blood samples, cytokine measurements were obtained from 456 samples. For PBMCs, samples from 401 and 466 individuals were available for cytokine measurements after 1 day and 7 days, respectively.

In vitro validation experiments

Study population and ethics statement
All studies with human blood samples were conducted in the Radboud University Nijmegen Medical Centre and the use of healthy volunteers was approved by the institutional ethics review board. All healthy volunteers donated blood samples after providing written informed consent.

Isolation of peripheral blood mononuclear cells (PBMC) and platelets
PBMCs were isolated from buffy coats isolated from healthy volunteers (Sanquin Bloodbank, Nijmegen, the Netherlands) using differential density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and adjusted to 3×10^6 cells/ml. To minimise platelet contamination, the PBMC pellet was washed five times with normal saline, which was two additional washing steps in comparison with the commonly-used PBMC isolation protocol. The PBMC to platelet ratio after the five washing steps was approximately two PBMC to one platelet. PBMC were suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA, USA) enriched with 10 μg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate.
Washed platelets were isolated by centrifugation of whole blood from healthy volunteers anticoagulated with 3.2 % sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) for 15 minutes (min) at 156 g without brake to obtain platelet-rich plasma (PRP). PRP was further supplemented with 1/10 acid citrate dextrose (ACD) and centrifuged twice at 330 g without brake for 15 min. The pellets were subsequently resuspended in HEPES tyrode (HT) buffer with a pH of 7.2. Prostacyclin (PGI2) 10 ng/ml was added to inhibit platelet activation after the isolation procedure. Platelets were let to rest for 30 min before being used in the experiments.

In vitro stimulation assays
PBMCs (3×10^6 cells/ml) were co-cultured with either washed platelets in a PBMC: platelet ratio of 1:150 or RPMI in round bottomed 96-well plates (Greiner, Alphen, Netherlands). Subsequently, LPS (6 ng/ml), *C. albicans* conidia (10^6 CFU/mL), and *S. aureus* (10^6/mL) were added. The requirement for direct interaction between PBMC and platelets was studied using a trans-well culture system (Costar 3413, Corning Life Sciences, Amsterdam, The Netherlands) in which PBMC and the stimuli were placed in the lower compartment. Lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) was purchased from Sigma and an extra purification step was performed as described previously (96). *C. albicans* conidia was heat-killed for 1 h at 100°C. *S. aureus* (ATCC 25923) was heat killed for 30 min at 100°C.
Supernatants were collected after 24 h of incubation at 37 °C, unless stated otherwise,

and stored at –20°C until cytokine levels were measured.

Cytokine measurements
The concentration of IL-1β, IL-6 and IFN-γ were measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA) (IL-1β: R&D Systems; IL-6, IFN-γ: Sanquin), according to the instructions of the manufacturer.

Platelet reactivity
Whole blood was collected from 6 consenting healthy volunteers by using venipuncture from the antecubital vein into citrate-anticoagulated tubes (3.2%; BD Vacutainer, Becton Dickinson). In addition, platelet-rich plasma (PRP) for selected experiments was obtained by centrifuging citrated-whole blood (n=6) for 15 minutes at 156 g without brake. Isolated PRP was pre-treated with recombinant IL-6, recombinant IL-1β, and recombinant IFN-γ (10-100 ng/ml) at 37°at 37060 minutes in 1.5 ml Eppendorf tubes (Eppendorf Biopur, Hamburg, Germany) and platelet reactivity to different platelet agonists was subsequently determined by flow cytometry. Pre-treatment was also performed with HEPES-buffered saline as control medium.
Platelet reactivity in PRP samples were determined by flow cytometric quantification of the platelet membrane expression of P-selectin (CD62P) and platelet-fibrinogen binding. In short, pre-treated samples were added to a mixture of HEPES-buffered saline and saturating concentrations of the following combinations of monoclonal antibodies: PE-labeled anti-CD62P (FITC-labeled Bio-Legend, San Diego, USA), FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK) and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). Platelets were activated with ADP (7.8 μM and 31.2 μM), CRP-XL (39 μM and 625 μM). After incubation for 20 minutes at room temperature, a fixative solution (0.2% paraformaldehyde) was added and samples were analyzed using an FC500 flow cytometer (Beckman Coulter, France). Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD61, which was defined as a median fluorescence intensity (MFI) exceeding the MFI of the matched isotype control. Next, the MFI of CD62P and fibrinogen on CD61-positive events were determined.

Statistical analysis
For the in vitro experiments, results from 6 biological replicates (n=6 PBMC donors) were pooled and dataare provided as mean with standard error of the mean (SEM). When values were below the detection limit of the ELISA, the corresponding detection limit was used. Differences between groups were analyzed with Student's t-tests. All analyses were carried out using Graphpad Prism software (La Jolla, CA, USA). The level of significance was set at $P< 0.05$.

Chapter 3

Differential effects of platelets and platelet inhibition by ticagrelor on TLR2– and TLR4-mediated inflammatory responses

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Abstract

Platelets and platelet-monocyte interaction play an important role in inflammation. Both pro- and anti-inflammatory effects of platelet inhibition have been reported in animal models. This study aimed to investigate the effect of platelets and platelet inhibition by the new $P2Y_{12}$ receptor antagonist ticagrelor on monocyte function, as assessed by cytokine responses to Toll-like Receptor (TLR) ligands. In a set of *in vitro* experiments, peripheral blood mononuclear cells (PBMC) incubated with the TLR2 ligand Pam3CSK4 produced less cytokines in the presence of platelets, whereas platelets increased the production of cytokines when PBMC were exposed to TLR4 ligand lipopolysaccharide (LPS). These effects of platelets were dependent on direct platelet-leukocyte aggregation and for the Pam3CSK4-induced response, on phagocytosis of platelets by monocytes. In a double blind, placebo-controlled crossover trial in healthy volunteers, a single oral dosage of 180 mg ticagrelor reduced platelet-monocyte complex (PMC) formation. This was associated with an increase in pro-inflammatory cytokines in blood exposed to Pam3CSK4, but a decrease in these cytokines in blood exposed to LPS. These findings show that platelets differentially modulate TLR2- and TLR4-mediated cytokine responses of PBMC. Through inhibition of platelet-leukocyte interaction, $P2Y_{12}$ receptor antagonists may either exert a pro- or anti-inflammatory effect during infections depending on the TLR primarily involved.

Keywords
Platelets, ticagrelor, inflammation, Toll-like receptor, platelet-monocyte complex

Introduction

Platelets are increasingly recognized to constitute an important part of the inflammatory response and host defense, apart from their role in hemostasis (1, 2). Activated platelets release an array of proteins with immune modulatory properties, including cytokines and chemokines, which enhance the activation of leukocytes and/or have a direct bactericidal effect (1). Furthermore, activated platelets form complexes with circulating leukocytes. This interaction is primarily mediated by the binding of P-selectin (CD62P) on the platelet membrane to its counter-ligand on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1) (3). The formation of platelet-monocyte complexes has been reported to enhance the activation status of both leukocytes and platelets (3-7).

The inflammatory function of platelets and their interaction with monocytes are especially interesting in view of the increasing use of platelet function inhibitors in prevention of cardiovascular events. The class of $P2Y_{12}$ receptor antagonists, such as the thienopyridines, clopidogrel, prasugrel and the most recently developed ticagrelor, are of special interest because they have been shown to reduce P-selectin expression on platelets and as such the formation of platelet-leukocyte complexes (8, 9). Interestingly, in the PLATelet inhibition and patient Outcomes (PLATO) trial, the lower overall mortality in patients treated with ticagrelor compared to clopidrogrel was partly the result of a lower death rate from pulmonary adverse events and sepsis (10).

The effects of platelets and platelet inhibition on the immune response may also depend on the type of pathogen involved. Pretreatment of mice with clopidogrel reduced the pro-inflammatory response following exposure to the Toll like receptor (TLR)-4 ligand LPS (11-13), but increased the inflammatory response in an arthritis model in rats induced by the peptidoglycan polysaccharide from group A streptococci, which is primarily a TLR2 ligand (14). This suggests that antiplatelet drugs may differentially influence the host defence against pathogens that primarily ligate TLR2 or TLR4.

We hypothesized that differences in platelet responses and platelet-monocyte complex formation to TLR2 and TLR4 ligands influence the inflammatory response by leukocytes and that this also explains the opposing effects of $P2Y_{12}$ inhibitors on the inflammatory responses in the animal studies outlined above. We therefore studied *in vitro* whether platelets differently influence the inflammatory response of monocytes after stimulation with TLR2 and TLR4 ligands. We subsequently explored the potential role of platelet inhibition in modulating this opposite inflammatory effects. In a randomized placebo-controlled crossover study, healthy volunteers were exposed to a single dose of ticagrelor and *ex vivo* cytokine production in whole blood stimulated by LPS and the TLR2/TLR1 ligand Pam3CSK4 challenge were determined in relation to platelet-monocyte complex formation.

Materials and methods

In vitro studies

Isolation of peripheral blood mononuclear cells (PBMC) and platelets
PBMCs were isolated from EDTA-anticoagulated blood from healthy volunteers using differential density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and adjusted to $3x10^6$ cells/ml. To minimize platelet contamination, the PBMC pellet was washed five times with normal saline. The PBMC to platelet ratio after the five washing steps was approximately two PBMC to one platelet. PBMC were suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA) enriched with 10 µg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate.
Washed platelets were isolated by centrifugation of whole blood anticoagulated with 3.2% sodium citrate (Becton Dickinson) for 15 minutes at 156g without brake to obtain platelet-rich plasma (PRP). PRP was further supplemented with 1/10 acid citrate dextrose (ACD) and centrifuged twice at 330g without brake for 15 minutes. The pellets were subsequently resuspended in HEPES tyrode (HT) buffer with a pH of 7.2. Prostacyclin (PGI_2) 10 ng/ml was added to inhibit platelet activation after the isolation procedure. Platelets were let to rest for 30 minutes before being used in the experiments. Frozen-fragmented platelets (FFP) were obtained by freeze-thawing of washed platelets for 3 cycles. Washed platelets, in the concentration of $4.5x10^8$ platelets/ml in RPMI, were subjected to 3 cycles of freeze-thawing to produce frozen fragmented platelets (FFP). In selected experiments, FFPs were treated with 10 U/ml RNAse A/T1 (Invitrogen, Carlsbad, CA) for 1 hour at 37°C.

In vitro stimulation assays
PBMCs ($3x10^6$ cells/ml) were co-cultured with either washed platelets in a PBMC: platelet ratio of 1:150 or 1:50, FFP ($4.5x10^8$ fragmented cells/ml) or RPMI in round bottomed 96-well plates (Greiner, Netherlands). After one hour at 37°C, Pam3CSK4 (6 µg/ml) or LPS (6 ng/ml) were added. Supernatants were collected after 24 hrs of incubation at 37°C, unless stated otherwise, and stored at -20°C until cytokine levels were measured.
The requirement for direct interaction between PBMC and platelets was studied using a trans-well culture system (Costar 3413, Corning Life Sciences, Amsterdam, The Netherlands) in which PBMC and the TLR ligands were placed in the lower compartment. Phagocytosis of platelets by monocytes was inhibited by pre-incubation of PBMC with cytochalacin B (1 µg/ml) at 37°C for 30 minutes.

Measurement of cytokine concentrations
The concentration of pro-inflammatory cytokines in culture supernatant was measured using commercially available ELISAs for tumor necrosis factor (TNF)-α and IL-1β (both from R&D, Abingdon, UK) and for IL-6 and interferon (IFN)-γ (both from Sanquin, Amsterdam, The Netherlands) according to the instructions of the manufacturers.

Platelet-monocyte complexes (PMC) and TLR2 and TLR4 expression by flow cytometry
PMC were measured by adding hepes-buffered saline with FITC-labeled anti-CD42b (anti-glycoprotein (GP)-1bα; Bio-Legend, San Diego, USA) and PE-labeled anti-CD14 (a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein; Bio-Legend, San Diego, USA) to 50 ul of whole blood (*ex vivo* study) or the PBMC-platelet cell suspension (*in vitro* studies). After 30 minutes incubation, Optilyse B (Beckman Coulter, Fullerton, CA) was added to lyse erythrocytes. Monocytes were identified and gated based on their CD14 positivity. Platelet–monocyte complex formation was determined by quantifying CD14+ cells that were also positive for the platelet identification marker CD42b. For the expression of TLR2 and TLR4 expression on the membrane of monocytes, PBMCs were incubated with anti-TLR2-Alexa-488 or anti-TLR4-Alexa-488 monoclonal antibodies (eBioscience, San Diego, CA), respectively and the expression of TLR2 and TLR4 was determined on CD14+ cells. Analyses of all flow cytometry experiments were carried out using a FC500 flow cytometer with CXP software (Beckman Coulter, Fullerton, CA, USA) within 1 h.

Platelet phagocytosis by monocytes
PRP was incubated with 5 uM of CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Life Technologies) for 45 minutes at 37°C for internal labelling of platelets. Excess CMFDA dye was removed by washing platelets with PBS and the platelet density was adjusted to $4.5x10^8$ platelets/ml in RPMI. PBMCs were isolated as previously described. The CMFDA-labelled platelet solution was co-incubated with PBMC coated on a six wells flat bottom plate in the presence of Pam3CSK4 (6 ug/ml), LPS (6 ng/ml) or RPMI for 90 min at 37°C. Uningested platelets were removed by treating the PBMC with 0.5mmol/L EDTA (Versene; Life Technologies) for 30 minutes at 37°C, followed by detachment of the PBMCs using PBS. PBMC were pelleted at 200*g* for 5 minutes and incubated with PE-labelled anti-CD14 to identify monocytes and the surface platelet marker PC7-labelled anti-CD61. After washing of the PBMCs with PBS platelet phagocytosis by monocytes was determined by flow cytometry. The gating strategy was set to separate monocytes with ingested platelets ($CMFDA^+/CD61^-$) from monocytes with platelets bound to the surface ($CMFDA^+/CD61^+$). The percentage of phagocytosis was calculated as the proportion of monocytes with ingested platelets in the total number of monocytes analyzed.

TLR2 and TLR4 mRNA expression

RNA was extracted from PBMC co-incubated with or without platelets and TLR ligands for 4 hrs using TRIzol (Sigma, St. Louis, MO). Chloroform and 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. After washing the preparation with 75% ethanol, the RNA was dissolved in 50 ul of diethylpyrocarbonate water. Isolated RNA was reverse-transcribed into complementary DNA using iScript cDNA synthesis kit (Bio-Rad). Relative expression of RNA was determined using the Step-one Plus (Applied Biosystems) real-time PCR apparatus with SYBR Green (Applied Biosystems). Real-time quantitative PCR data were corrected for expression of the housekeeping gene human *B2M*. The following primers were used (5'-3'): GAATCCTCCAATCAGGCTTCTCT (forward) and GCCC TGAGGGAATGGAGTTTA (reverse) for TLR2, GGCATGCCTGTGCTGAGTT (forward) and CTGCTACAACAGATACTACAAGCACACT (reverse) for TLR4, and ATGAGTATGCCTGCCGTGTG (forward) and CCAAATGCGGCATCTTCAAAC (reverse) for β_2 microglobulin (B2M) (Biolegio, The Netherlands). Relative mRNA expression of the target genes were calculated by correcting the Ct-values of respective genes with the Ct-value of the housekeeping gene β_2 microglobulin and using the formula of $2^{-\Delta Ct \times 1000}$.

Ex vivo study

Design

Seven healthy male volunteers, aged 19-26 years who did not use any medication were enrolled in a randomized, double blind, placebo-controlled crossover study. The volunteers received a single oral dose of 180 mg ticagrelor followed by a placebo two weeks later or vice-versa (supplemental data figure 1). Whole blood was collected from the cubital vein in citrate and lithium-heparin tube (BD Vacutainer, USA) two hours after administration of the study drug. The regional medical ethics committee (CMO Regio Arnhem-Nijmegen) approved the study protocol, and informed consent was obtained from all healthy volunteers. This study was registered at www.clinicaltrials.gov (NCT01996735).

Whole blood stimulation and platelet-monocyte complex formation

For the whole blood stimulation, heparinized whole blood was diluted 1:5 with RPMI 1640 and incubated in 24-well plates for 24 hours at 37°C with 120 ug/ml Pam3CSK4 (EMC Microcollections, Tübingen, Germany) and 100 ng/ml LPS (*E. coli* serotype O55:B5; Sigma Chemical Co. St. Louis, MO), which was used after an extra purification step as previously described (15). Cytokines were measured in the culture supernatant by ELISA. Platelet-monocyte complexes were determined by flow cytometry on citrate anticoagulated blood before and after one hour of co-incubation with Pam3CSK4 and LPS, as described below.

Flow cytometry-based platelet function test

Inhibition of ADP-induced platelet reactivity was determined using a flow cytometry-based platelet function test, of which the details have been described previously (16, 17). In summary, sodium citrate (3.2%) anticoagulated venous blood was incubated with PE-labeled anti-CD62P antibodies and FITC-labeled anti-CD42b antibodies (Bio-Legend, San Diego, USA) or FITC-labeled anti-fibrinogen (Dako, Glostrup, Denmark) and PE-labeled anti-CD42b antibodies (Bio-Legend, San Diego, USA) and two concentrations (7.8 and 31.2 uM) of the platelet agonist adenosine diphosphate (ADP). After incubation for 20 minutes at room temperature, 0.2% paraformaldehyde was added as fixative. Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD42b and the mean fluorescence intensity (MFI) of CD62P and fibrinogen on CD42b positive events was measured.

Statistical analysis

Differences in the *ex vivo* study were analyzed using the Wilcoxon signed-rank tests for non-parametric data. For the *in vitro* experiments, results from three sets of experiments (involving n≥9 PBMC donors, unless otherwise specified) were pooled and data are provided as mean with standard error of the mean (SEM). Differences between groups were analysed with Student's t-tests. All analyses were carried out using Graphpad Prism software (La Jolla, CA, USA). The level of significance was set at $p<0.05$.

Results

Platelets have opposite effects on cytokine production after stimulation with Pam3CSK4 or LPS

We exposed isolated PBMCs to Pam3CSK4 or LPS for 24 hrs in the presence or absence of washed isolated platelets and measured cytokines in the supernatants. While platelets significantly increased concentrations of IL-1β and IL-6 in LPS exposed samples, the opposite was found in Pam3CSK4 exposed samples (Figure 1). Addition of platelets to Pam3CSK4 exposed PBMCs resulted in a strong and general downregulation of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα and the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1Ra) (IL-1Ra data presented in supplemental data figure 2). In addition, LPS stimulation of PBMC in the presence of platelets also led to the suppression of IL-10. Pam3CSK4 or LPS did not induce a significant increase in IFN-γ concentrations (supplemental data figure 2). Addition of platelets to PBMC

without TLR ligands or incubation of platelets with LPS or Pam3CSK4 did not lead to detectable IL-1β or IL-6 release. The modulating effects of platelets also did not require intact platelets as frozen-fragmented platelets (FFP) elicited the same responses as intact washed platelets (supplemental data figure 3).

Figure 1

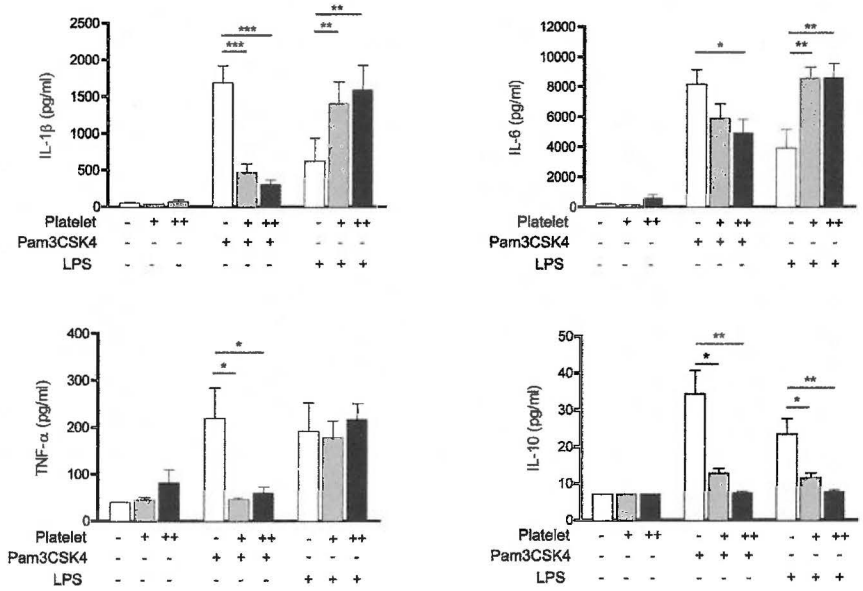


Figure 1. Differential effects of platelets on cytokine production by peripheral blood mononuclear cells (PBMC) following exposure to Pam3CSK4 or LPS.

PBMC were co-incubated with LPS or Pam3CSK4 for 24 hrs in the absence and presence of washed platelets. The panels show concentrations of the pro-inflammatory cytokines interleukin (IL-)1β, IL-6 and tumor necrosis factor (TNF)-α and the anti-inflammatory cytokine IL-10 in the culture supernatant. The concentrations are expressed as mean with standard error of mean (SEM) from at least 3 experiments using 3 PBMC donors each. + 50 platelets for every PBMC, ++ 150 platelets for every PBMC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Role of direct platelet-leukocyte interaction and platelet phagocytosis

Adhesion of washed platelets to monocytes and platelet phagocytosis were measured by flow cytometry after one hour of co-culturing of PBMC with washed platelets in the presence of either Pam3CSK4 or LPS. Exposure to Pam3CSK4 resulted in a strong increase in platelet adhesion to monocytes and in a significant increase in platelet phagocytosis (Figure 2; gating strategies described in supplemental data figure 4), as previously described (18). Platelet phagocytosis could be reduced by adding the actin polymerization inhibitor cytochalasin B. In contrast, LPS did not lead to a detectable increase in PMC formation or in platelet phagocytosis.

Figure 2

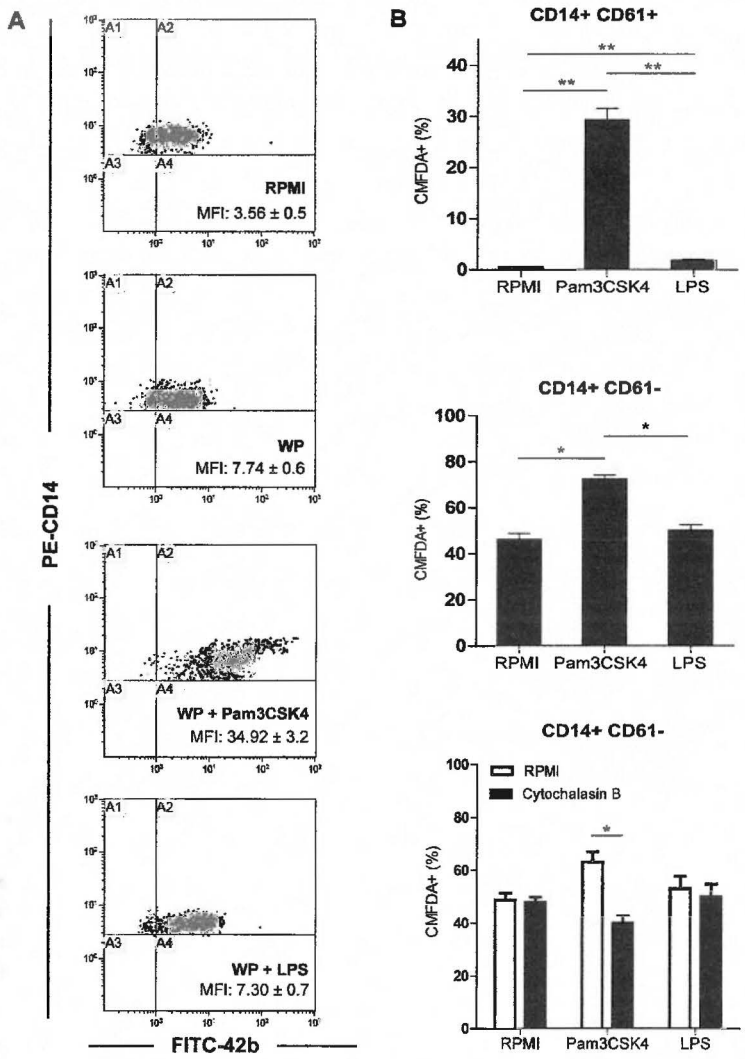


Figure 2. Platelet-monocyte complex (PMC) formation and platelet phagocytosis by monocytes following exposure to Pam3CSK4 or LPS.

PBMC were co-cultured with washed platelets for one hour in the presence of Pam3CSK4 or LPS. PMC formation was determined by flow cytometry and expressed as the mean fluorescence intensity (MFI) of the platelet marker CD42b on CD14 positive cells (Panel A). Phagocytosis of platelets by monocytes was also determined using flow cytometry, Monocytes with ingested platelets were defined as CD14 positive cells that were positive for the internal platelet label CMFDA, but negative for the platelet surface marker CD61 (Panel B).. Cytochalasin B was used to inhibit platelet phagocytosis. Data presented are means with SEM from at least 3 experiments. Additional details with optical scatter plots of PMC formation and platelet phagocytosis are presented in supplemental data figure 4. RPMI, culture medium; WP, washed platelets; PBMC, peripheral blood mononuclear cells. * $p < 0.05$, ** $p < 0.01$.

To study whether inhibition of PMC formation and platelet phagocytosis would reverse the observed changes in cytokine concentrations, we first repeated the PBMC incubation with Pam3CSK or LPS in the presence and absence of platelets in a transwell system, in which the PBMC and platelets were physically separated. This abolished both the attenuating effects of platelets on Pam3CSK4-induced cytokine production and the enhancing effects on LPS-induced cytokine production (Figure 3A). We next studied the effect of inhibiting platelet phagocytosis by monocytes. Cytochalasin B reversed the attenuating effects of platelets on cytokine production following Pam3CSK4 stimulation, but did not reverse the stimulating effects of platelets upon LPS stimulation (Figure 3B). Cytochalasin B has also been reported to prevent platelet activation (19) and inhibition of platelet-monocyte interaction might have contributed to the reversal of the platelet-mediated attenuation of cytokine responses to Pam3CSK4. However, no reduction in platelet-monocyte interaction was observed using cytochalasin B in our *in vitro* system (supplemental data figure 5).

Figure 3

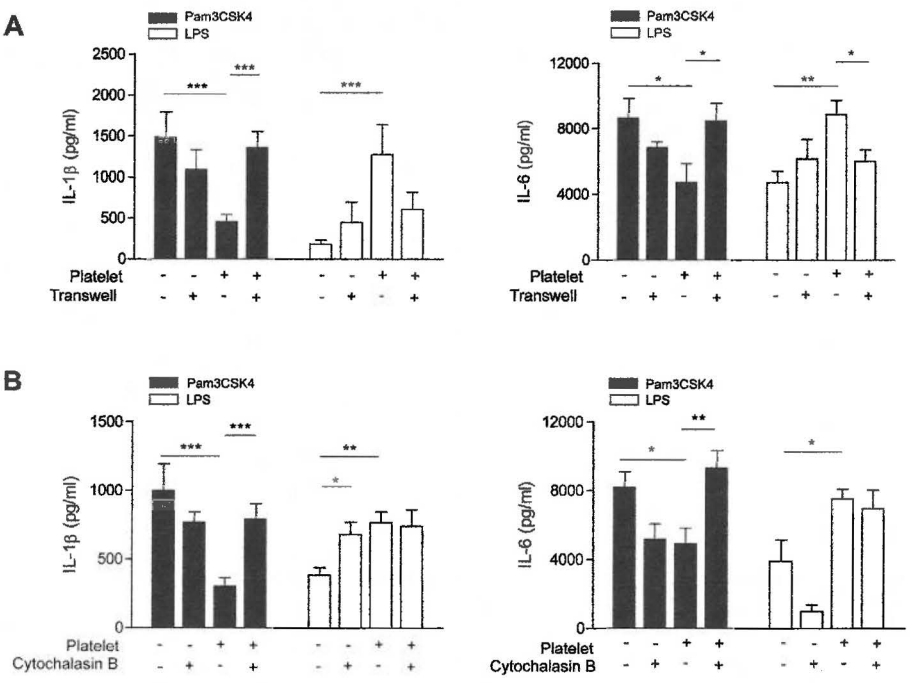


Figure 3. Changes in IL-1β and IL-6 production by PBMC by physical separation of PBMC and platelets and by inhibition of platelet phagocytosis.

PBMCs were co-cultured for 24 hours with washed platelets and Pam3CSK4 or LPS with or without physical separation of platelets and PBMC using a Transwell system (Panel A) or in the presence or absence of cytochalasin B, which inhibits phagocytosis (Panel B). Interleukin (IL)1β and IL-6 concentrations were determined in the culture supernatant. Platelets were added in a ratio of 150 platelets for every PBMC. Presented data are means with SEM from at least 3 experiments. PBMC, peripheral blood mononuclear cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 4

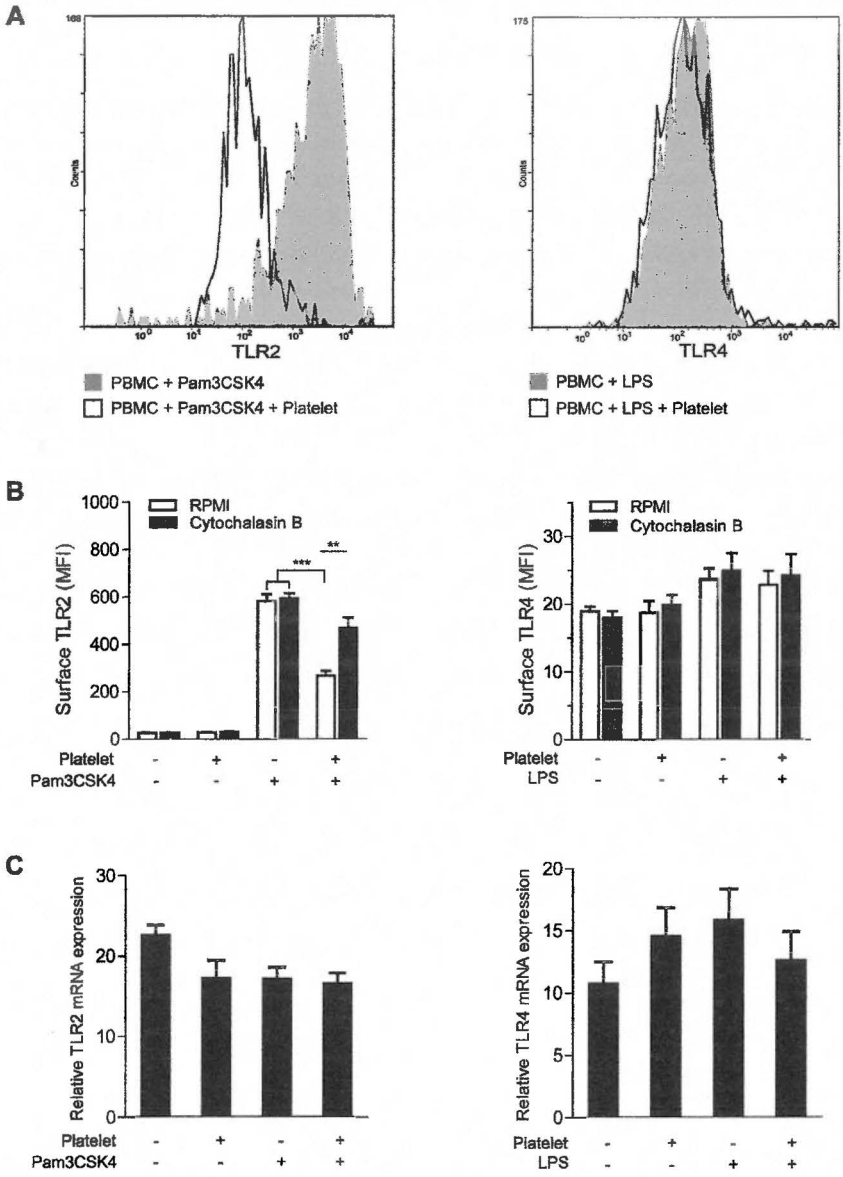


Figure 4. Platelet-induced change in monocyte surface expression and mRNA expression of Toll-like receptor (TLR)2 and TLR4.

PBMC were co-cultured with or without platelets and Pam3CSK4 or LPS for 1 hour. Surface expression of TLR2 and TLR4 on CD14 positive cells was measured by flow cytometry. Cytochalasin B was used to inhibit platelet phagocytosis (Panel A and B). PBMC were co-cultured with or without platelets and Pam3CSK4 or LPS for four hours. TLR2 and TLR4 mRNA expression was determined by real-time PCR. The relative TLR2 and TLR4 mRNA expression was calculated by correcting the Ct-values for the Ct-value of the housekeeping gene β_2 microglobulin (Panel C). Presented data are means with SEM from at least 3 experiments. PBMC, peripheral blood mononuclear cells. ** $p < 0.01$, *** $p < 0.005$.

Changes in TLR membrane expression and transfer of functional platelet RNA

We next explored mechanisms through which PMC formation and platelet phagocytosis modulate cytokine responses to TLR ligands. First, we investigated whether platelet binding and phagocytosis changed the expression of TLR2 and TLR4 on monocytes. Exposure of PBMC to Pam3CSK4 in the presence of platelets for one hour resulted in a significant decrease in TLR2 expression (Figure 4 panel A). In contrast, there was no change in TLR4 expression following exposure to LPS in presence of platelets. Cytochalasin B reversed the decline of surface TLR2 expression after stimulation with Pam3CSK4 (Figure 4 panel B), while there was no change in TLR2 mRNA expression (Figure 4 panel C), suggesting that internalization during platelet phagocytosis was responsible for the decreased expression.

Another possible mechanism through which platelets may influence monocyte function is the transfer of functional RNA from phagocytosed platelets to monocytes. Frozen-fragmented platelets were incubated with RNAse A/T1 prior to co-culture with PBMC and Pam3CSK4 or LPS. This resulted in a complete reversal of the decrease in IL-1β upon Pam3CSK4 stimulation, but it had no effect on IL-6 concentrations (Figure 5). RNAse A/T1 also had no effect on platelet-induced cytokine production upon LPS stimulation.

Figure 5

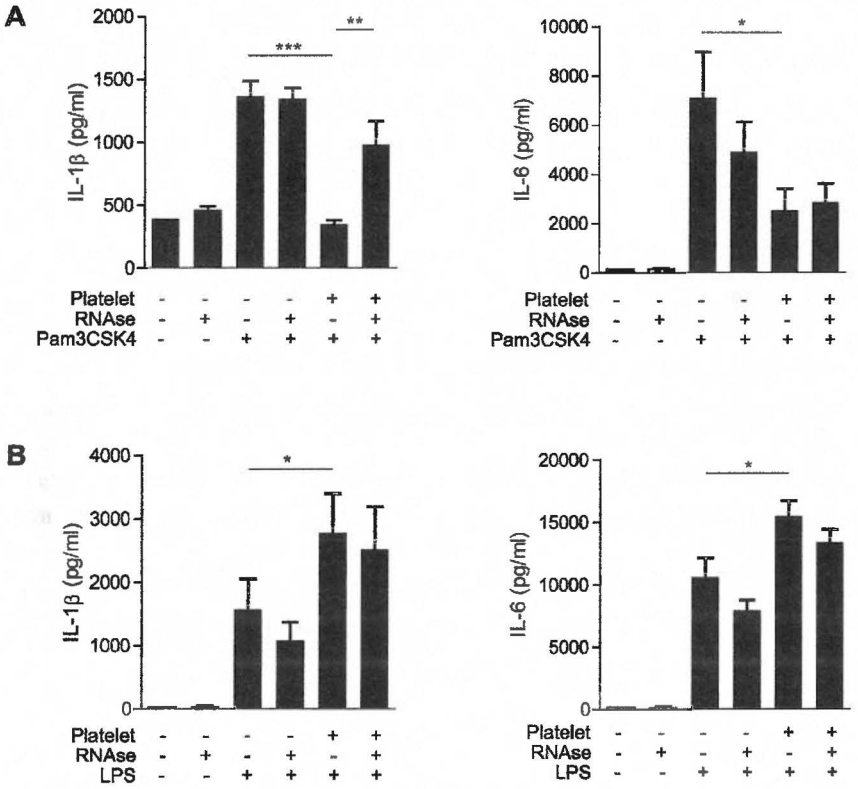


Figure 5. Effect of degradation of platelet RNA on platelet-induced changes in cytokine production by PBMC.

Frozen-fragmented platelets (FFP) or FFP pre-incubated with RNAse A/T1 to degrade platelet RNA were co-incubated with PBMC and Pam3CSK4 (Panel A) or LPS (Panel B) for 24 hours. IL-1β and IL-6 concentrations were measured in the culture supernatant. Presented data are means with SEM. PBMC, peripheral blood mononuclear cells. * $p < 0.05$.

Ticagrelor has opposite effects on cytokine production in LPS- and Pam3CSK4-stimulated whole blood and reduces platelet-monocyte complexes

$P2Y_{12}$ receptor antagonists are known to reduce PMC formation and we therefore determined the effect of ticagrelor on the observed platelet-mediated immune effects. In a cross-over study, seven healthy male volunteers were given a single dose of 180 mg ticagrelor or placebo. As expected, intake of ticagrelor resulted in a full inhibition of ADP-induced platelet P-selectin expression and platelet-fibrinogen binding (Figure 6A). Blood of the volunteers was collected two hours after the administration of placebo or

ticagrelor for whole blood stimulation with Pam3CSK4 or LPS and determination of PMC formation. Data on LPS stimulation in one volunteer were missing. In agreement with the findings from the *in vitro* experiments above, ticagrelor had opposite effects on cytokine production by PBMC (Figure 6B). In six of seven volunteers, ticagrelor induced an increase in the concentration of IL-1β and IL-6 in the supernatant of Pam3CSK4 stimulated samples with median (IQR) values increasing from 98.4 pg/ml (89.9 to 245.7 pg/ml) to 227.9 pg/ml (179.1 to 867.1 pg/ml; P=0.031) and from 6.0 ng/ml (2.1 to 29.7 ng/ml) to 21.2 ng/ml (3.5 to 68.4 ng/ml; P=0.016) for IL1β and IL-6, respectively. In contrast, ticagrelor resulted in a reduction in the concentrations of these cytokines in LPS stimulated samples with median IL-1β and IL-6 concentrations decreasing from 2,231 pg/ml (1,337 to 2,890 pg/ml) to 1,799 pg/ml (1,039 to 2,289 pg/ml; P=0.048) and from 59,9 ng/ml (36.4 to 71 ng/ml) to 36,7 ng/ml (28.1 to 53.4 ng/ml; P=0.035), respectively.

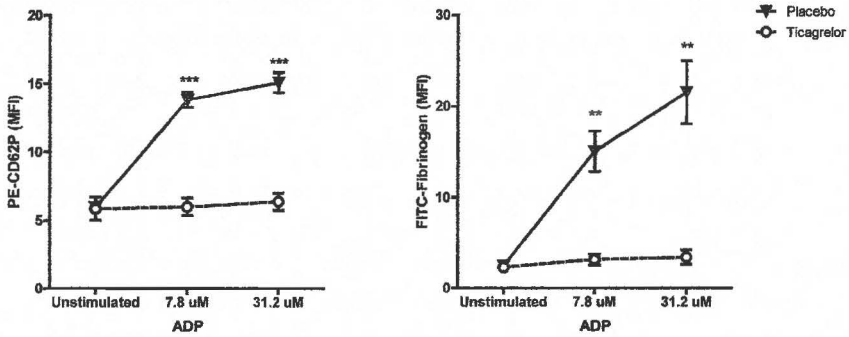
PMC were measured before and after one hour stimulation. Pam3CSK4 stimulation resulted in pronounced PMC formation, with roughly 10-fold higher mean fluoresence intensity (MFI) of the platelet marker CD42b on gated monocytes. In contrast, LPS stimulation did not lead to a detectable increase in PMC formation. A significant decline in PMC formation compared with placebo was observed after ticagrelor in unstimulated and Pam3CSK4 and LPS stimulated samples (Figure 6C).

Figure 6. Ticagrelor-induced changes on *ex vivo* whole blood cytokine production and PMC formation upon stimulation by Pam3CSK4 or LPS.

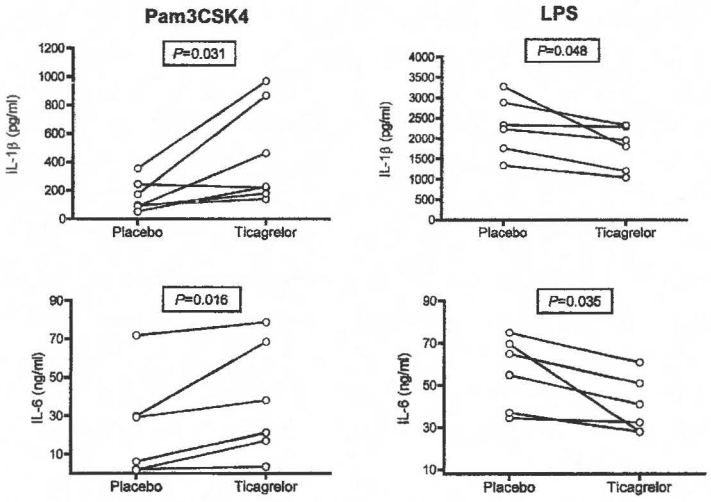
Seven healthy volunteers received a single dose of ticagrelor or placebo in a cross over trial. Whole blood was taken two hours after intake of ticagrelor or placebo, and platelet reactivity was determined within 30 minutes of blood drawing. Whole blood was incubated with FITC- or PE-labeled anti-CD42b antibodies as platelet identification marker and PE-labeled anti-CD62P (P-selectin) antibodies or FITC-labeled anti-fibrinogen as markers of platelet degranulation and activation of the fibrinogen receptor, respectively. The expression of P-selectin and platelet-fibrinogen binding following incubation with a low (7.8 uM) and high (31.2 uM) concentration of the platelet agonist ADP was determined by flow cytometry (Panel A) Whole blood was stimulated for 24 hours by Pam3CSK4 or LPS. *Ex vivo* IL-1β and IL-6 were measured in the culture supernatant (Panel B). PMC formation was determined by flow cytometry and expressed as the mean fluorescence intensity (MFI) of the platelet marker CD42b on CD14 positive cells. PMC was determined two hours after intake of ticagrelor or placebo and one hour after start of whole blood stimulation by Pam3CSK4 or LPS. Data on LPS stimulation from one volunteer were missing (Panel C). Presented data are means with SEM. Asterisks correspond to statistical differences with baseline unstimulated samples. ** $p < 0.01$, *** $p < 0.005$.

Figure 6

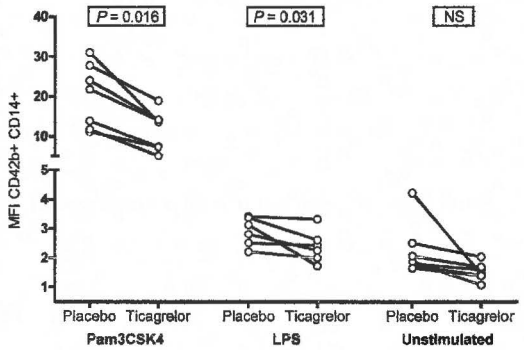
A. Platelet inhibition by ticagrelor



B. Whole blood cytokine production



C. Platelet-monocyte complexes



Discussion

In the present study, we show that platelets have different effects on the inflammatory response by leukocytes, depending on whether TLR2 or TLR4 ligands stimulate the cells. Platelets attenuated the production of pro-inflammatory cytokines following PBMC stimulation with the TLR2/TLR1 ligand Pam3CSK4, whereas they increased production of these cytokines following stimulation with the TLR4 ligand LPS. The effects of both stimulants were dependent of direct contact between platelets and PBMC. Pam3CSK4 strongly induced PMC formation, in contrast to LPS, and induced platelet phagocytosis by monocytes. Phagocytosis of platelets was a necessary step for the downregulation of cytokine production in Pam3CSK4-stimulated monocytes.

The potential clinical relevance of these findings is that platelet inhibition may influence the immune response during infections and that the direction of this influence may differ depending on the micro-organism and the TLR pathway involved. The $P2Y_{12}$ receptor antagonists have become a cornerstone of antiplatelet therapy for patients with acute coronary diseases. In line with the findings from our *in vitro* experiments, we found that ticagrelor administered to healthy volunteers increased pro-inflammatory cytokines in whole blood stimulated *ex vivo* with Pam3CSK4, while it decreased cytokines in LPS-stimulated blood, along with a reduction in PMC formation. So far, data from clinical studies in humans on the effects of $P2Y_{12}$ receptor antagonists on the susceptibility and outcome of infections are limited. Clopidogrel has been reported to increase the risk for surgical site infections and bacteremia following coronary artery bypass surgery (20), as well as community acquired pneumonia, but its use was also associated with a more favorable outcome in pneumonia and sepsis (13, 21). In the PLATO trial, ticagrelor was associated with lower mortality following pulmonary adverse events and sepsis compared with clopidogrel (10).

Our observations are in accordance with previous studies demonstrating activation of monocytes by interaction with platelets (4, 7, 22, 23). Activated platelets express P-selectin, which ligates with monocyte PSGL-1, inducing nuclear translocation of nuclear factor-kappa B (NF-kB) and the NF-kB-dependent inflammatory genes (4, 22). This mechanism likely contributes to the increased cytokine responses to LPS by addition of platelets. The protein CD14 mediates recognition of LPS by TLR4 and addition of recombinant or purified CD14 in our system might have further increased platelet responses to LPS. Whereas LPS failed to induce platelet phagocytosis, Pam3CSK4 resulted in strong PMC formation and platelet phagocytosis. How Pam3CSK4-induced platelet phagocytosis leads to downregulation of cytokine production needs further study. We found that degradation of platelet RNA by RNAse A/T1 prevented the decrease in monocyte cytokine production. Platelets contain a diverse and complex mRNA and microRNA repertoire (24) which can be transferred to nucleated cells (18).

Which platelet microRNAs or RNA transcripts might be responsible for the general downregulation in cytokine production requires additional studies and was beyond the scope of our current work. Another possible mechanism was the decrease in surface TLR2 expression on monocytes following co-incubation of PBMC with platelets and Pam3CSK4. Inhibition of platelet phagocytosis using cytochalasin B prevented this decrease in TLR2 expression which makes shielding of TLR2 by attached platelets less likely. Our group previously reported internalization of TLR2 following phagocytosis of conidia of *Aspergillus fumigatus* (25) and we speculate that internalization of TLR2 during platelet phagocytosis is responsible for these observations. Recently, Gudbrandsdottir and colleagues (26) also reported that activated platelets have anti-inflammatory properties. However, opposite to our findings, platelets also reduced TNFα production by LPS stimulated monocytes. The fact that thrombin-receptor-activating peptide (TRAP) was used in their experiments to stimulate platelets may explain these seemingly discordant results.

Our present findings also provide a mechanistic explanation for the seemingly contrasting results on the effects of platelets and the use of platelet antagonists on inflammatory responses in animal studies. Whereas both thrombocytopenia and clopidrogel therapy have been reported to decrease inflammatory responses in response to LPS in mice (11-13, 27), clopidogrel increased inflammation to cell wall components of group A *streptococcus*, which ligate TLR2 (14).

No other $P2Y_{12}$ inhibitors beyond ticagrelor were included in our study and our observation therefore cannot be automatically translated to the other members of this class. This is especially relevant since ticagrelor has a dual mode of action through inhibition of not only the $P2Y_{12}$ receptor but also adenosine uptake (28). As adenosine is a well-known modulator of inflammation (29), its upregulation via equilibrative nucleoside transporter (ENT)-1 inhibition may also have contributed to our observations. On the other hand, inhibition of PMC formation, which was a necessary step for the platelet-induced effects on cytokine production, has been described for multiple $P2Y_{12}$ antagonists (9, 30) and appears to be a class effect of $P2Y_{12}$ inhibitors in general.

The limitations of our study include the relatively low number of healthy volunteers who were exposed to ticagrelor and placebo. The crossover design, however, in which participants are their own controls allows us to draw statistical valid conclusions with such a limited number of participants. Secondly, as mentioned above, no other $P2Y_{12}$ inhibitors beyond ticagrelor were used. Thirdly, cytokine responses were determined *ex vivo* in whole blood. While experimental human endotoxemia models are available, *in vivo* challenges using TLR2 ligands or other Gram-positive ligands are not available to our knowledge. Fourthly, neutrophil activation and platelet-neutrophil complex formation were not addressed in our study, principally because IL-6 and Il-1β are predominantly produced by activated monocytes in the circulation. Still, platelet-neutrophil complexes

may also influence the host immune response to infections.
In conclusion, platelets and platelet-leukocyte interaction augment TLR4-mediated responses by leukocytes, whereas those mediated by TLR2 are attenuated. Through the inhibition of platelet-monocyte complex formation, $P2Y_{12}$ receptor antagonists can either exert a pro- or anti-inflammatory effect in infections depending on the TLR primarily involved.

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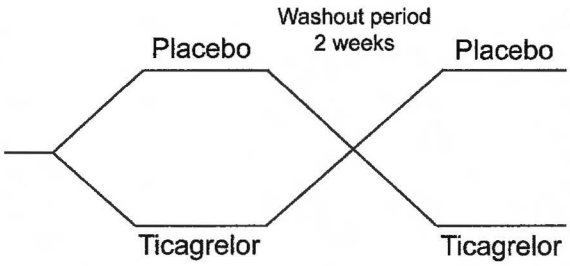
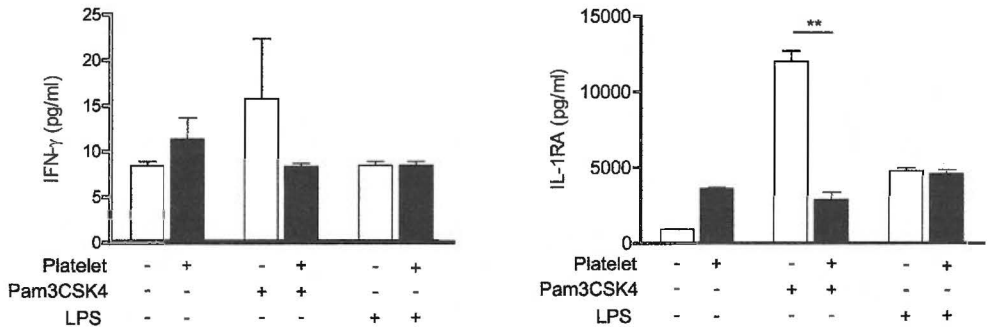
Supplemental data figures

Supplemental data figure 1.



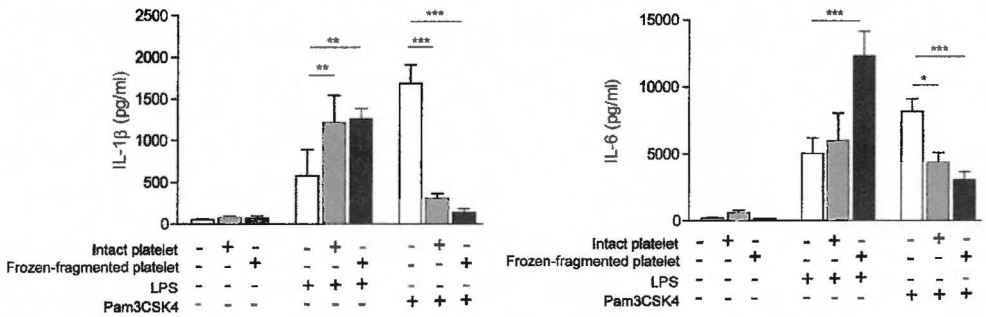
Supplemental data figure 1. Design of the *ex vivo* study.
Seven healthy volunteers received a single oral dose of 180 mg ticagrelor followed by a placebo two weeks later or vice-versa.

Supplemental data figure 2.



Supplemental data figure 2. Platelet-induced changes in production of interferon (IFN)-gamma and interleukin-1 receptor antagonist (IL-1RA) by peripheral blood mononuclear cells (PBMC) upon exposure to Pam3CSK4 or LPS.
PBMC were co-incubated with LPS or Pam3CSK4 for 24 hrs in the absence and presence of washed platelets (ratio PBMC : platelet of 1:50). The panels show concentrations of IFN-γ and IL-1RA in the culture supernatant. The concentrations are expressed as mean with standard error of mean (SEM) from at least 3 experiments using 3 PBMC donors each. ** $p<0.01$.

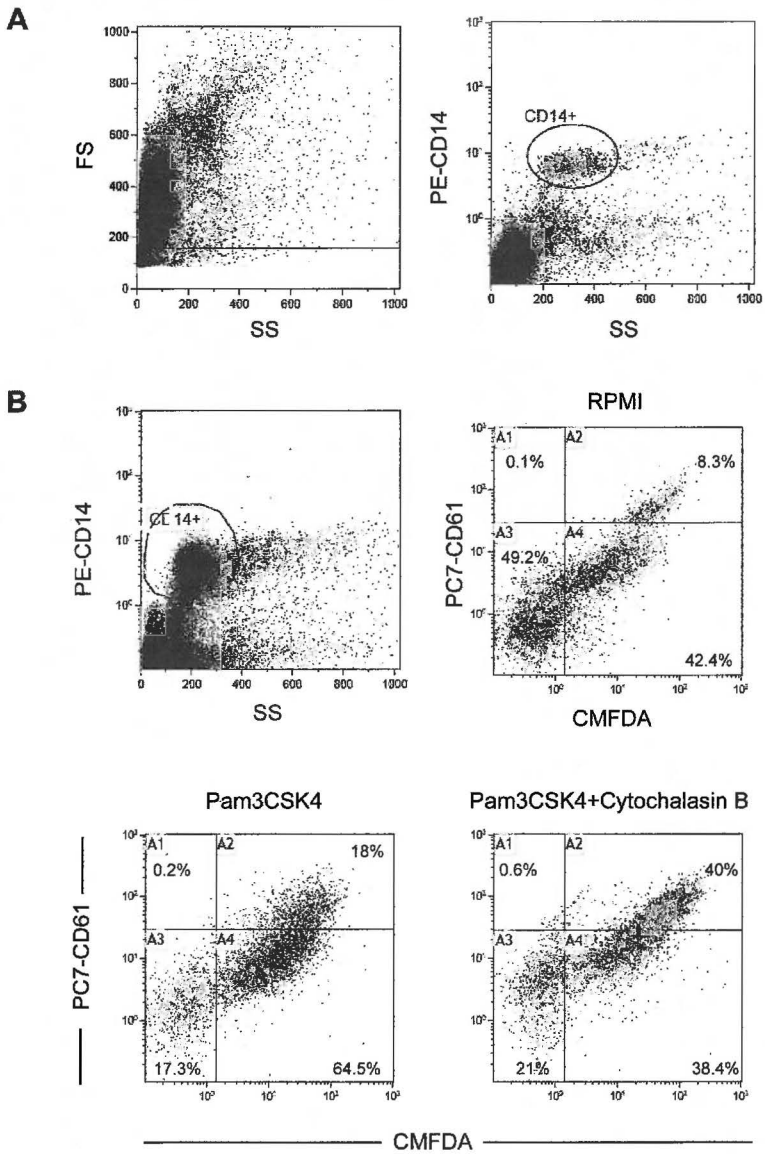
Supplemental data figure 3.



Supplemental data figure 3. Interleukin(IL)-1β and IL-6 production by PBMC stimulated by Pam3CSK4 or LPS in presence of either intact washed platelets or frozen-fragmented platelets (FFP).

PBMCs were co-cultured for 24 hours with intact platelets or FFP and Pam3CSK4 or LPS IL-1β and IL-6 concentrations were determined in the culture supernatant. Presented data are means with SEM from at least 3 experiments using 3 PBMC donors each. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

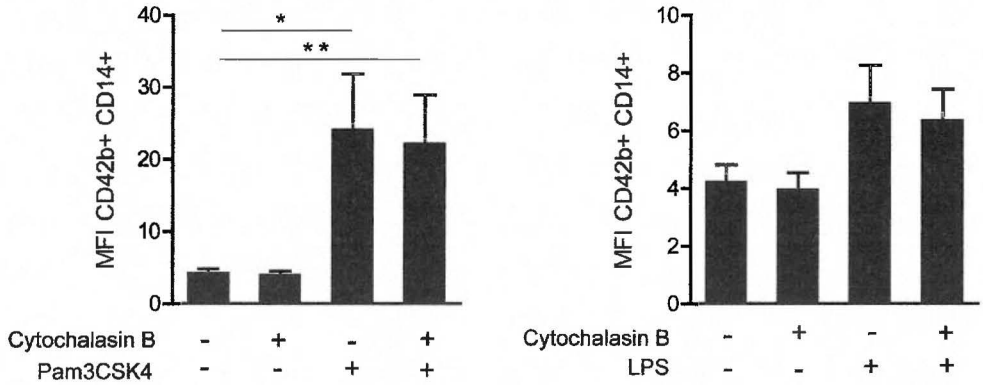
Supplemental data figure 4.



Supplemental data figure 4. Gating strategy and optical scatter plots for PMC formation and platelet phagocytosis (as shown in figure 2).

Platelets were incubated with CD42b antibodies (for determination of PMC) or CD61 antibodies together with the internal platelet label CMFDA (for determination of platelet phagocytosis). PBMCs were co-incubated for one hour with washed platelets and Pam3CSK4 or LPS. Gating strategy for PMC formation with optical side (FS, X axis) and forward scatter (SS, Y axis) plot of PBMC and gating of monocytes by CD14 positivity (Panel A). For platelet phagocytosis, monocytes were first gated based on CD14 positivity. Monocytes with platelets inside were defined as CMFDA positive/PC7-CD61 negative cells; while monocytes with surface-bound platelets were identified by being PC7-CD61 positive. Cytochalasin B was used to inhibit platelet phagocytosis (Panel B).

Supplemental data figure 5.



Supplemental data figure 5. Cytochalasin B does not modulate PMC formation.
PBMC were co-incubated with washed platelets for 1 hour in presence or absence of cytochalasin B. PMC formation was determined by flow cytometry and expressed as the mean fluorescence intensity (MFI) of the platelet marker CD42b on CD14 positive cells.

Chapter 4

Human recombinant alkaline phosphatase inhibits *ex vivo* platelet activation in humans

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Abstract

Sepsis-associated acute kidney injury (AKI) is associated with high morbidity and mortality. Excessive platelet activation contributes to AKI through the formation of microthrombi and amplification of systemic inflammation. Two phase II trials demonstrated that bovine-intestinal alkaline phosphatase (AP) improved renal function in critically ill patients with sepsis-associated AKI. In this study, we characterized the platelet-inhibiting effects of a human recombinant AP. Whole blood and platelet-rich plasma (PRP) of healthy volunteers (n=6) was pre-treated *ex vivo* with recAP, whereafter platelet reactivity to ADP, collagen-related peptide (CRP-XL) and Pam3CSK4 was determined by flow cytometry. RecAP (40 U/ml) reduced the platelet reactivity to ADP (inhibition with a median of 47%, interquartile range 43-49%; $P<0.001$) and tended to reduce platelet reactivity to CRP-XL (9%, 2-25%; $P=0.08$) in whole blood. The platelet-inhibiting effects of recAP were more pronounced in PRP both for ADP- (64%, 54-68%; $P=0.002$) and CRP-XL-stimulated samples (60%, 46-71%; $P=0.002$). RecAP rapidly converted ADP into adenosine, whereas antagonism of the A2A adenosine receptor partially reversed the platelet inhibitory effects of recAP. Platelets of septic shock patients (n=5), who showed 31% (22-34%; $P=0.03$) more pronounced reactivity compared to healthy volunteers, was completely reversed by recAP treatment. In conclusion, we demonstrate that recAP inhibits *ex vivo* human platelet activation through dephosphorylation of ADP and formation of adenosine as its turnover product. RecAP is able to reverse the platelet hyperreactivity present in septic shock patients. These effects may contribute to the beneficial effects of recAP as a new therapeutic candidate for sepsis-associated AKI.

Keywords: platelet activation, antiplatelet agents, sepsis, acute kidney injury, alkaline phosphatase

Introduction

The morbidity and mortality of severe sepsis and septic shock remains high despite advances in medical care (1). A frequent consequence of sepsis is the development of acute kidney injury (AKI), which increases mortality to >50% and progresses to chronic kidney disease in up to one-third of critically ill patients (2, 3). New therapies to improve the prognosis of sepsis and sepsis-associated AKI patients are therefore urgently needed. An example of a promising novel therapy is the dephosphorylating enzyme alkaline phosphatase (AP). AP is a membrane-bound homodimeric glycoprotein of which four isozymes are known: placental, germ cell, intestinal, and tissue-nonspecific (liver/bone/kidney) (4). In two phase II trials, bovine-derived AP was shown to have a renal protective effect in critically ill patients with sepsis-associated AKI (5, 6).
Subsequently, a human recombinant AP (recAP) was developed with important advances over bovine-derived AP in terms of immunity, stability and enzymatic activity (7). This novel chimeric enzyme was developed by replacing the crown domain of a human intestinal AP with the crown domain of human placental AP. How exactly AP exerts its beneficial effects is still incompletely understood. *In vitro* studies indicate that the anti-inflammatory effects of recAP is related to its dephosphorylating activity on lipopolysaccharide (LPS), which is involved in the inflammatory cascade observed in sepsis patients, and the conversion of the pro-inflammatory molecule adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into tissue-protective adenosine (4, 8-10). ADP is one of the most potent platelet activators which interacts with the P2Y12 and P2Y1 receptors. P2Y12 is the major target of platelet inhibitory drugs such as clopidogrel, prasugrel and ticagrelor (11, 12). Platelets themselves also contain large quantities of ATP and ADP in dense granules, which are released when they are activated. In contrast, adenosine inhibits platelet activation through the A2A and A2B adenosine receptor (13).
Three studies from the late 1980s showed that bovine-derived kidney AP inhibited platelet aggregation (14-16), but since then, the platelet-modulating effects of AP has not received much attention. Nevertheless, these effects may be relevant as accumulating evidence supports a key role for platelets in sepsis and subsequent kidney damage. First, excessive platelet activation during sepsis leads to platelet sequestration and platelet-rich microthrombi, blocking the microcirculation and thereby compromising the kidney function. Second, activated platelets release an array of proteins with immune modulatory properties that contribute to the amplification of systemic inflammation, and form complexes with leukocytes, altering their phenotype (17-19).
The aims of our study were to investigate the effects of recAP on platelet activation, identify involved pathways with special emphasis on ADP and adenosine, and determine the effect of recAP on sepsis-associated platelet hyperreactivity.

Methods

Materials

Human recombinant alkaline phosphatase (recAP) and inactivated recAP were kindly provided by AM-Pharma (Bunnik, The Netherlands). Adenosine 5'-diphosphate (ADP) was purchased from Sigma-Aldrich (St Louis, MO, USA), cross-linked collagen-related peptide (CRP-XL) was a generous gift from Prof. dr. R. Farndale (Cambridge, UK). Pam3CSK4 (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)- (2*RS*)-propyl] -[*R*]- cysteinyl-[*S*]-seryl- [*S*]- lysyl- [*S*]- lysyl-[*S*]-lysyl-[*S*]-lysine × 3 HCl) was purchased from InvivoGen (San Diego, CA, USA). The A2A adenosine receptor (AR) antagonist ZM-241385 and A2BAR antagonist PSB-1115 were purchased from Tocris Bioscience (Bristol, UK). HEPES-buffered saline was used as control medium.

Platelet function assays

Platelet reactivity

Whole blood was collected from 6 consenting healthy volunteers for the *ex vivo* experiments by using venipuncture from the antecubital vein into citrate-anticoagulated tubes (3.2%; BD Vacutainer, Becton Dickinson). In addition, platelet-rich plasma (PRP) for selected experiments was obtained by centrifuging citrated-whole blood (n=6) for 15 minutes at 156 g without brake, and platelet concentration was adjusted to $1x10^8$/ml. Whole blood or PRP was pre-treated with different concentrations of recAP (10-100 U/ml) at 37°C for 45 minutes in 1.5 ml Eppendorf tubes (Eppendorf Biopur, Hamburg, Germany) and platelet reactivity to different platelet agonists was subsequently determined by flow cytometry. Pre-treatment was also performed with HEPES-buffered saline, inactivated recAP (in protein concentration equivalent to recAP) and potato apyrase as controls.

Platelet reactivity was determined by flow cytometric quantification of the platelet membrane expression of P-selectin (CD62P) and platelet-fibrinogen binding, which correspond with platelet activation and aggregation, respectively, as previously described (20, 21). In short, pre-treated samples were added to a mixture of HEPES-buffered saline and saturating concentrations of the following combinations of monoclonal antibodies: PE-labeled anti-CD62P (FITC-labeled Bio-Legend, San Diego, USA), FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK) and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). Platelets were activated with ADP (7.8 µM and 31.2 µM), CRP-XL (39 µM and 625 µM) or Pam3CSK4 (60 ug/ml). After incubation for 20 minutes at room temperature, a fixative solution

(0.2% paraformaldehyde) was added and samples were analyzed using an FC500 flow cytometer (Beckman Coulter, France). Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD61, which was defined as a median fluorescence intensity (MFI) exceeding the MFI of the matched isotype control. Next, the MFI of CD62P and fibrinogen on CD61-positive events were determined.
To study the washed-out effect of recAP on platelet membrane proteins, PRP was exposed to recAP (40 U/ml) at 37°C for 45 minutes, samples were centrifuged for 15 minutes at 330 g without brake to pellet platelets, and the supernatant was removed. Pelleted platelets were subsequently resuspended in fresh-autologous plasma to its original PRP volume and platelet reactivity was determined. In experiments where the adenosine receptor antagonists were used, whole blood and PRP was co-incubated with ZM-241385 and PSB-1115 (30 μM) for 15 minutes prior to recAP pre-treatment at 37°C.

Platelet-monocyte complex formation
Platelet-monocyte complex formation leads to reciprocal activation of both cells and is regarded as a sensitive marker of platelet activation (22, 23). Platelet-monocyte complex formation was determined by adding whole blood samples to HEPES-buffered saline mixture containing saturating concentrations of PE-labelled anti-CD14 (a glycosyl-phosphatidylinositol (GPI)-linked membrane glycoprotein; Biolegend) and PC7-labelled anti-CD61 as monocyte and platelet markers, respectively. After 20 minutes of incubation at room temperature, Optilyse B (Beckman Coulter, CA, USA), a lysing solution which also contains 3.4% formaldehyde, was added to lyse erythrocytes. Monocytes were gated based on CD14 positivity. Platelet-monocyte complex formation was determined by quantifying the MFI of CD61 on the CD14-positive cells.
In our experiments, whole blood was incubated for 45 minutes at 37°C in the presence or absence of recAP. Platelets may be pre-activated by *ex vivo* handling prior to the start of the experiments or by the use of lysing buffer. We therefore determined the extent of platelet activation in whole blood with or without 45 minutes of incubation in 37°, and whether differential platelet activation occurred when Optilyse B was used as a lysing buffer.

Measurement of purine content

In a set of control experiments using PRP from 4 healthy volunteers, adenosine, AMP, ADP and ATP content was determined by HPLC. In brief, PRP were pre-treated for 45 minutes in 37 with medium, recAP (40 U/ml) or inactivated recAP (in equivalent protein content). Subsequently, platelet activation was induced by incubation with ADP

or CRP-XL for 20 minutes in room temperature, and samples were centrifuged for 15 minutes at 330 g without brake to obtain the supernatant. Thereafter, four volumes of supernatant (400 µl) were mixed with 1 volume of chloroacetaldehyde (6x diluted in 1M acetate buffer, pH 4.5; Sigma-Aldrich, Zwijndrecht, Netherlands), followed by derivatization (60 min, 70 °C, 500rpm) and centrifugation (3 min, RT, 13400 rpm), whereafter the supernatant was transferred to a HPLC vial and injected. Purines were separated by HPLC system (Thermo Scientific, Illinois, USA) using a Polaris C18-A column (150 x 4.6 mm) with gradient elution using eluent A (0.1M K_2HPO_4, 10 mM TBAHS (pH 6.5), and 2% MeOH) and eluent B (H_2O: ACN: THF; 50:49:1). Retention times were 7.1 (adenosine), 8.4 (AMP), 12.5 (ADP), 16.2 (ATP) and 14.8 min (cAMP). Quantification was based on peak areas of the samples and reference standards measured with fluorescence (excitation and emission wavelengths set at 280 and 420 nm, respectively).

Ex vivo study of sepsis patients

A total of five patients with septic shock according to the definitions stated by ACCP and SCCM consensus conference (24) admitted to the Intensive Care Unit of the Radboud university medical center (Radboudumc) were enrolled between July 2014 and February 2015. Exclusion criteria were the use of any medication which could affect platelet count and function. The study was carried out in accordance with the applicable rules concerning the review of the local ethical committee and informed consent. Citrate-anticoagulated whole blood was collected on day 1, 3, 5 and 7 and was directly pre-treated with recAP (40 U/ml) or HEPES-buffered saline as a control at 37°C for 45 minutes. Platelet reactivity to ADP (31 uM) and CRP-XL (39 uM), as well as platelet-monocyte complex formation were measured by flow cytometry, as previously described.

Statistical analyses

Data are expressed as medians with interquartile range (IQR). This is an exploratory study and non-parametric statistical analyses was selected due to the small sample size. For the experiments with healthy volunteers, differences between multiple groups were tested using the Kruskall-Wallis test with Dunn's post hoc test. Data from the patient study were analyzed using the Mann-Whitney U test. Statistical analyses were performed using Graphpad Prism 6 (San Diego, USA). $P < 0.05$ was considered statistically significant.

Results

RecAP inhibits *ex vivo* platelet activation in whole blood of healthy volunteers

The extent of which recAP inhibits platelet activation was determined by pre-treatment of whole blood with recAP, followed by stimulation with the platelet agonists ADP (31 μM) and CRP-XL (39 μM). Whole blood samples pre-treated with 40 U/ml of recAP demonstrated an impairment in ADP-induced platelet reactivity with the median (interquartile range [IQR]) maximum inhibition of 47% (43-49%; *P*=0.0009) in P-selectin expression on platelets, while the reactivity to CRP-XL stimulation was reduced to a lesser extent (9%, 2-25%; *P*=0.08). Similar trends were observed in the platelet-fibrinogen binding (Figure 1A; representative histograms are presented in Figure 1B, with gating strategies described in Suppl. Figure 1). We explored the use of increasing concentrations and pre-treatment duration of recAP, as well as different concentrations of ADP and CRP-XL. Increasing the recAP concentration beyond 40 U/ml or the pre-treatment duration beyond 45 minutes did not further augment the inhibition of platelet activation and these aforementioned conditions were therefore chosen for subsequent experiments (Suppl. Figure 2).

Platelet-monocyte complex formation induced by ADP and CRP-XL was strongly inhibited by recAP (Figure 1C). We additionally tested the platelet response to immunological stimulation by using Pam3CSK4 (60 μg/ml), a synthetic agonist of Toll-like-receptor 2 (TLR2). RecAP also demonstrated strong inhibition of Pam3CSK4-induced platelet expression of P-selectin, platelet-fibrinogen binding and platelet-monocyte complex formation (Suppl. Figure 3).

Pre-activation of unstimulated platelets may occur by *ex vivo* handling prior to the start of experiments. However, in our setting, the percentage of platelets expressing P-selectin between samples with and without 45 minutes of incubation in 37° were comparable (4.3%, 3.3-4.7% vs. 4.1%, 3.2-4.7%; *P*=0.8). Whole blood samples fixated and lysed with Optilyse B for the measurement of platelet-monocyte complex also did not show increased platelet pre-activation when compared with the 0.2% paraformaldehyde-fixated samples (data not shown).

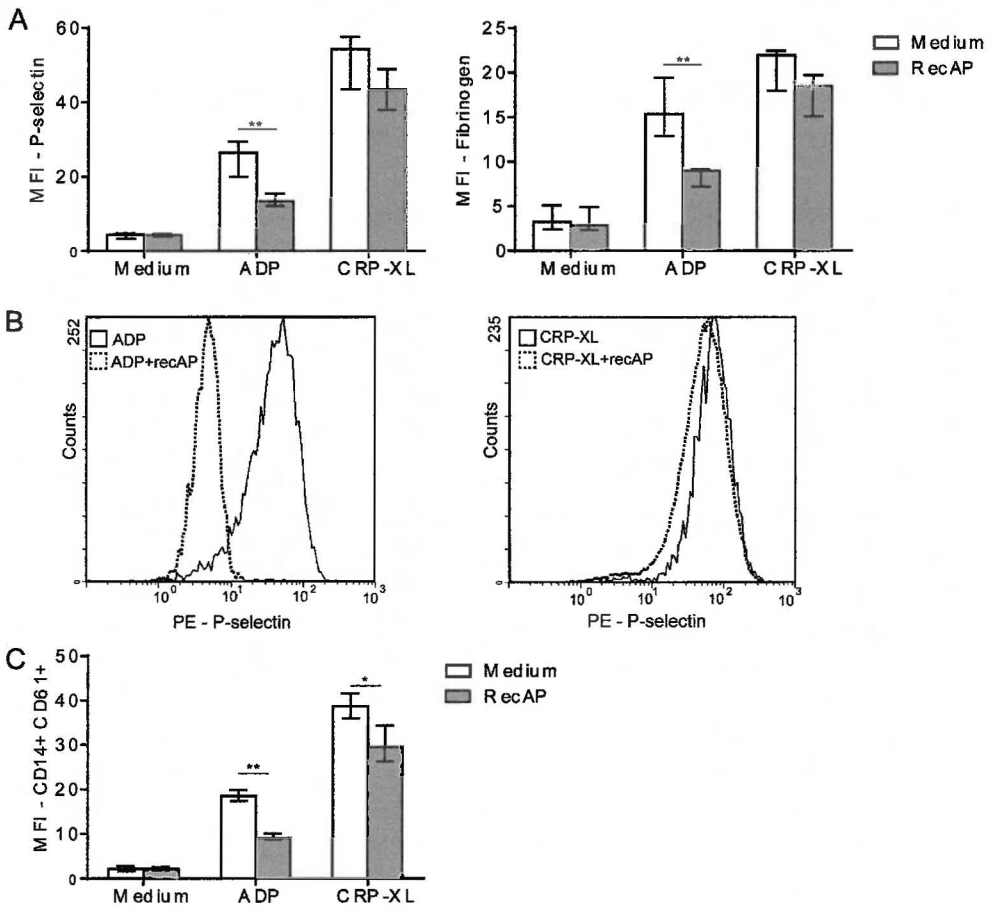

Figure 1. RecAP inhibits platelet activation by ADP and CRP-XL.
(A) Whole blood from healthy volunteers was pre-treated with medium or recAP (40 U/ml) for 45 minutes and subsequently stimulated with the platelet agonists adenosine diphosphate (ADP; 31 μM) and collagen-related peptide (CRP-XL; 39 μM). The median fluorescence intensity (MFI) of the platelet surface expression of P-selectin and platelet-fibrinogen binding was measured by using flow cytometry. (B) Representative histograms for ADP and CRP-XL stimulation shown. (C) Platelet-monocyte complex formation after exposure to ADP and CRP-XL was determined by quantifying the MFI of the platelet marker CD61 on CD14+ cells. Data are presented as medians with interquartile range (IQR) from 6 healthy donors. * $P < 0.05$, ** $P < 0.001$.

The effect of recAP is dependent on its dephosphorylating activity

To determine whether the enzymatic dephosphorylating activity of recAP is required for its platelet-inhibitory effect, whole blood was pre-treated with recAP or inactivated recAP, the latter having no enzymatic dephosphorylating activity (10), and subsequently stimulated with ADP and CRP-XL. No reduction of platelet P-selectin or platelet-fibrinogen binding was found in samples containing inactivated recAP (Figure 2A).

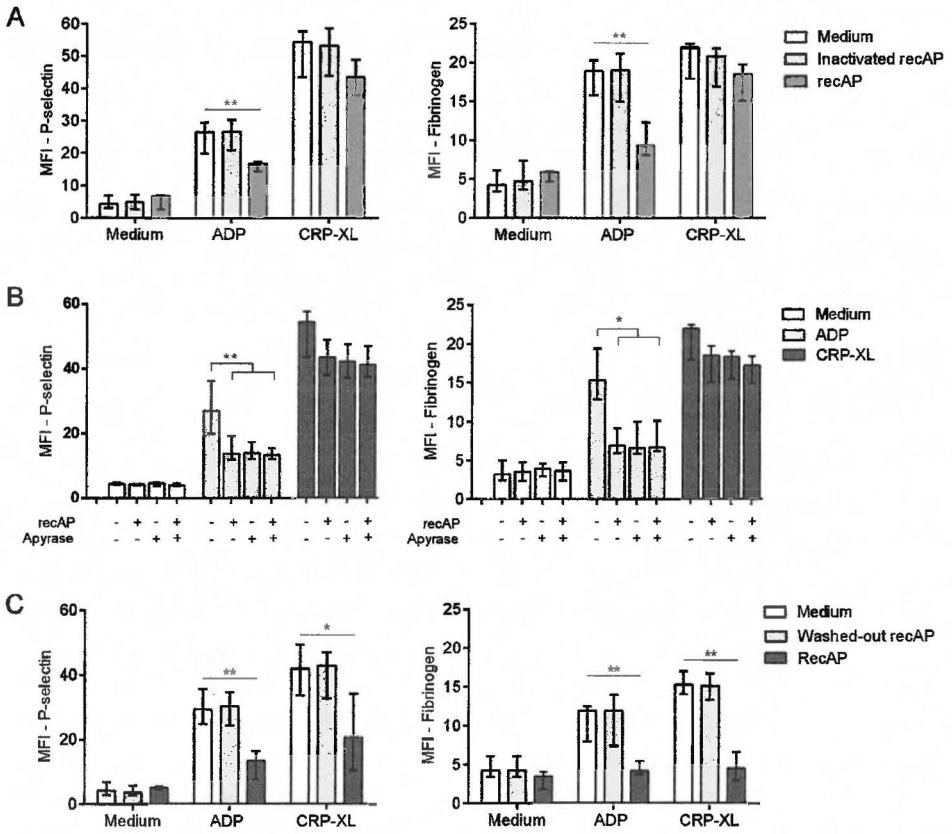

Figure 2. RecAP inhibition of platelet activation is dependent on its enzymatic dephosphorylating activity.

(A) Whole blood was pre-treated for 45 minutes with medium, recAP (40 U/ml) or inactivated recAP (in equivalent protein content) prior to stimulation with ADP (31 μM) or CRP-XL (39 μM). Platelet expression of P-selectin and binding with fibrinogen was determined by flow cytometry. (B) Whole blood was pre-treated with medium, recAP, apyrase (5 U/ml), or a combination of both recAP and apyrase, before exposure to ADP or CRP-XL. (C) Platelet-rich plasma (PRP) was pre-treated for 45 minutes with either medium or recAP. RecAP in the PRP samples was subsequently either washed-out, with the remaining platelets resuspended in fresh autologous platelet-poor plasma (washed-out recAP samples), or not (recAP samples). Samples are subsequently incubated with medium, ADP or CRP-XL. Data are presented as medians with IQR from 6 healthy donors. * $P < 0.05$, ** $P < 0.01$.

Next, we compared the effects of recAP with those of apyrase as both compounds demonstrate ADP-degrading activity. Apyrase is also known to inhibit ADP-dependent platelet aggregation (25, 26). Pre-treatment of whole blood with recAP and potato-derived apyrase had a comparable platelet-inhibiting effect on samples stimulated with ADP and CRP-XL (Figure 2B). The combination of both compounds had no synergistic effect, suggesting that both shared similar ADP-dependent mechanism and already fully inhibited ADP-dependent platelet activation.
A previous study suggested that AP might also exert its platelet-inhibitory effects through the dephosphorylation of platelet membrane proteins (15). To study this mechanism, we pre-treated PRP with recAP, thereby exposing the membrane of platelets to recAP. We subsequently isolated the platelets from PRP and resuspended them in fresh autologous platelet-poor plasma, thereby washing out any potentially remaining recAP. No platelet inhibition occurred once the recAP-containing plasma was washed out and replaced with fresh plasma, indicating that there is no persisting, residual dephosphorylating effect of recAP on the platelet membrane, and that the platelet-inhibiting activity of recAP is dependent on its presence in test medium (Figure 2C).

The effect of recAP is exerted directly on platelets

Leukocytes can secrete inflammatory mediators that activate platelets or the coagulation system. Furthermore, ADP can also alter the function of leukocytes, which in turn can affect platelet activation (9, 23). In order to explore the direct effect of recAP on platelets, we performed experiments on platelet-rich plasma (PRP). RecAP pre-treatment on PRP resulted in platelet inhibition of ADP-stimulated (64%, 54-68%; P=0.002). Interestingly, in PRP samples, those stimulated with CRP-XL showed significant platelet hyporeactivity after pre-treatment with recAP (60%, 46-71%; P=0.002), a finding not present in whole blood samples. These data confirmed that recAP acts directly on platelets. Similar to data from whole blood, inactivated recAP did not demonstrate any platelet-inhibiting effect, and potato-derived apyrase had a comparable inhibiting effect on both ADP- and CRP-XL-induced platelet activation in PRP (Figure 3). Data on the effect of different doses and pre-treatment duration of recAP on PRP is presented on Suppl. Figure 4.

Formation of adenosine contributes to the platelet-inhibiting effect of recAP

Next to a direct dephosphorylating effect of recAP on ADP, we studied whether increased adenosine formation may also contribute to the observed platelet-inhibiting effects. APs

are the only known ectonucleotidases which can degrade extracellular ATP, ADP and AMP into adenosine (9). Adenosine exerts both anti-inflammatory and tissue-protective effects, and signals by binding with 1 of its 4 receptors: A1AR, A2AAR, A2BAR or A3AR. Adenosine signaling via the A2AAR and A2BAR was reported to inhibit platelet activation (13, 27). In our experiments, blocking the A2AAR receptor by an A2AAR antagonist partially reversed the platelet inhibiting effects by recAP in whole blood (data not shown) as well as in PRP (Figure 4A). In contrast, the use of an A2BAR antagonist had no effect (data not shown). RecAP might convert ADP into AMP and adenosine, with the latter contributing its effect via the A2AAR. To confirm that adenosine was indeed generated by the turnover from ADP, we measured purine content from the supernantant of the PRP pre-treated with recAP or inactivated recAP that had subsequently been stimulated with ADP and CRP-XL. ADP-stimulated samples demonstrate increased adenosine levels when pre-treated with recAP (27-fold, 17-49-fold, P=0.02) with negligible ATP and ADP levels, indicating their full turnover into AMP and adenosine (Figure 4B). Interestingly, CRP-XL induced ADP release when compared to non-stimulated samples (11-fold [2-69-fold, P=0.02), which was completely removed upon pre-treatment with recAP (Suppl. Figure 5). Taken together, these data suggest that not only the dephosphorylation of ADP inhibits platelet activation and reactivity, but the concomitant formation of adenosine also augments the inhibitory effect of recAP on platelet function.

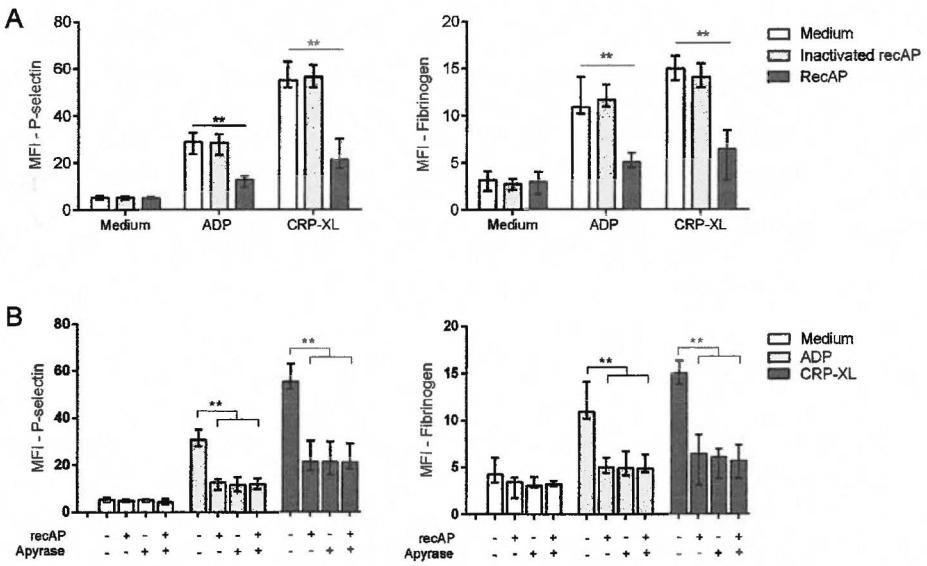

Figure 3. RecAP exerts its inhibitory activity directly on platelets.
(A) Platelet-rich plasma (PRP) was pre-treated for 45 minutes with medium, recAP (40 U/ml) or inactivated recAP (in equivalent protein content) prior to stimulation with ADP (ADP (31 μM) or CRP-XL (39 μM). Platelet expression of P-selectin and binding with fibrinogen was determined by flow cytometry. (B) PRP was pre-treated with medium, recAP, apyrase (5 U/ml), or a combination of both recAP and apyrase, before exposure to ADP or CRP-XL. Data are presented as medians with IQR from 6 healthy donors. ** $P < 0.01$.

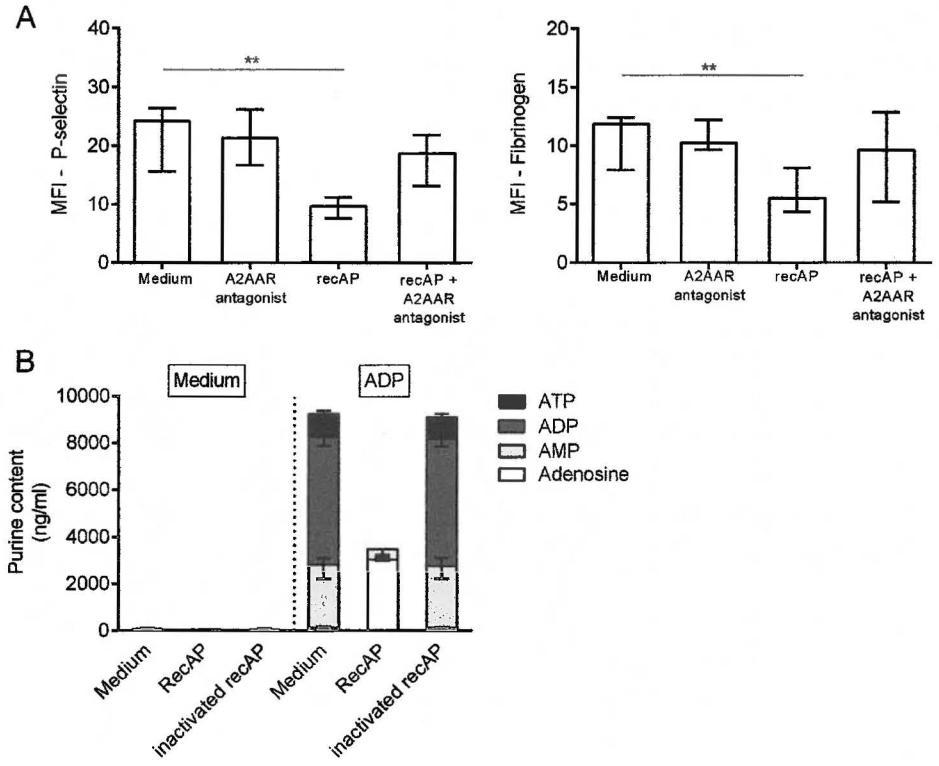

Figure 4. Adenosine formation contributes to the platelet-inhibiting activity of recAP.
(A) PRP was pre-treated with the selective A2AAR antagonist (ZM-241385; 30 uM) for 15 minutes, followed by incubation with recAP (40 U/ml). Platelet P-selectin (left panel) and fibrinogen binding to platelets (right panel) was determined by flow cytometry after stimulation with ADP (31 uM). Presented data are medians with IQR from 6 healthy donors. ** $P < 0.01$. (B) Purine levels were determined in the supernatant of PRP pre-treated with recAP (40 U/ml) or inactive recAP (in equivalent protein content) and subsequently stimulated with ADP (31 uM). Presented data are medians with IQR from 4 healthy donors.

Ex vivo exposure to recAP reverses sepsis-induced platelet hyperreactivity

We performed platelet function tests on whole blood of septic shock patients (n=5) collected on day 1, 3, 5 and 7 after the start of norepinephrine infusion, with or without *ex vivo* recAP pre-treatment. Patients' characteristics are described in Table 1. Patients demonstrated a significant increase of platelet activation and aggregation on day 1 compared to healthy controls which normalized upon measurement on subsequent days. Upon *ex vivo* pre-treatment with recAP, whole blood samples of these patients demonstrated reversal of the sepsis-associated platelet hyperreactivity as well as reduced platelet-monocyte complex formation (Figure 5).

Table 1. Patient characteristics

All patients (n = 5)	
Demographics	
M/F	4/1
Age (yrs)	59.8 ± 16.9
Body mass index (kg/m2)	26.9 ± 1.9
Acute Physiology and Chronic Health	22.6 ± 4.7
Evaluation II	
Systemic inflammatory response syndrome	
Temperature (°C)	
<36	-
36-38	37.6 [37.5-37.7]
>38	38.5 [38.5-38.6]
Leukocytes (x109/L)	
<4	-
4-12	7.1 [5.4-8.7]
>12	17.0 [12.8-17.7]
Heart rate (bpm)	108 ± 9
Patients on ventilator (%)	100
Platelet count (cellsx109/L)	
Day 1	222 ± 89
Day 3	171 ± 71
Day 5	175 ± 84
Day 7	308 ± 194

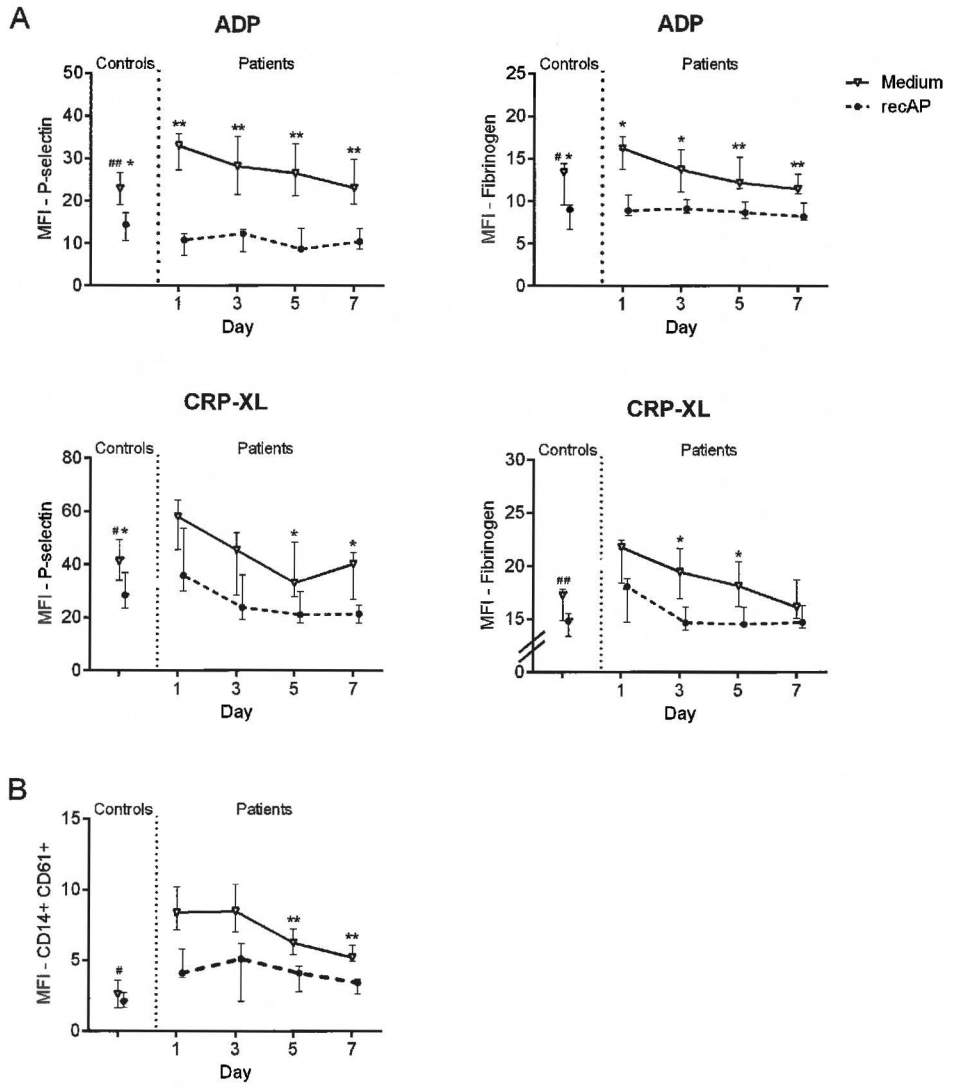

Figure 5. Platelet hyperreactivity in sepsis patients.
(A) A total of five septic shock patients were followed up on day 1, 3, 5 and 7. Whole blood was collected and pre-treated *ex vivo* with or without recAP (40 U/ml) and stimulated with ADP (31 uM) and CRP-XL (39 uM). Whole blood from healthy controls (n=6) was collected on a single time point. Platelet P-selectin and platelet-fibrinogen binding were measured by flow cytometry. (B) Platelet-monocyte complex formation was determined by quantifying the MFI of the platelet marker CD61 on CD14+ cells in whole blood. Data from 2 patients on day 1 and day 3 were missing. Presented data are medians with IQR. * $P < 0.05$ medium vs. recAP pre-treatment, ** $P < 0.01$ medium vs. recAP pre-treatment, medium vs. recAP pre-treatment, # $P < 0.05$ healthy controls vs. patient on day 1 (Mann-Whitney test).

Discussion

RecAP is a novel dephosphorylating enzyme currently under investigation as a new therapy for sepsis-associated AKI (clinicaltrials.gov, NCT02182440). Its exact mechanism of action is still incompletely understood. In the present study, we report that recAP reduces platelet reactivity to different platelet agonists in a threshold dose-fashion. RecAP strongly inhibited ADP-induced platelet activation in whole blood, and additionally CRP-XL-induced platelet activation in PRP. These data from PRP confirmed that recAP has a direct effect on platelets. Our data indicate that the dephosphorylation of ADP and the formation of adenosine as its turnover product are primarily responsible for the observed platelet-inhibiting effects. Additionally, exposure of whole blood of patients with septic shock to recAP reversed the sepsis-associated platelet hyperreactivity, further substantiating that this mechanism of action may be of importance in septic patients.

ADP directly activates platelets via the P2Y12 and P2Y1 receptors on the platelet membrane, resulting in degranulation and aggregation (11, 28). To determine whether the platelet-inhibiting effects of recAP also occurs on different platelet activation pathway, we studied the platelet reactivity to CRP-XL which activate platelets via their GPVI receptor (29). In addition to measuring platelet reactivity to the thrombotic stimulation by the aforementioned agonists, we examined the platelet reactivity to immunological stimulation by using Pam3CSK4, which induces platelet activation through TLR2 (30, 31). The release of endogenous ADP from platelet dense granules forms an essential amplification mechanism for platelet activation via both GPVI and TLR2 (29, 31), which can explain the broad platelet-inhibiting effects of recAP. RecAP resulted in a strong inhibition of CRP-XL-induced platelet activation in PRP but not in whole blood. Upon CRP-XL-stimulation, ADP release by platelets is known to provide an amplification mechanism for platelet activation (29). Indeed, our experiments confirm that CRP-XL induced the release of ADP from platelets. In addition, ADP can also be taken up by other blood cells like erythrocytes, monocytes and lymphocytes (32-34). RecAP exerts its effect through the dephosphorylation of ADP into adenosine; and we therefore speculate that the effect of recAP in whole blood is less striking because the relative abundance of ADP that is affecting platelets is lower in whole blood compared to PRP, thereby limiting the availability of adenosine. In addition, adenosine in whole blood is rapidly taken up by erythrocytes, neutrophils and lymphocytes (35). As a consequence, higher platelet inhibition by recAP observed in the CRP-XL-stimulated PRP samples compared to the whole blood samples is a plausible result. Furthermore, there seemed to be a more pronounced platelet inhibition in samples stimulated with the TLR2 agonist Pam3CSK4 compared to the platelet agonist ADP. As is the case with ADP and CRP-XL stimulation, the extent of platelet activation and consequently its

inhibition by recAP, in the Pam3CSK4-stimulated samples may be dose-dependent and as such, the percentage of reduction cannot be compared between the different stimuli. Our finding that antagonism of the A2A adenosine receptor partly reversed the platelet inhibition by recAP underlines the importance of adenosine generation in the effects of recAP. Adenosine is released from several different cells or generated from extracellular metabolism of ATP, ADP and AMP by different ectoenzymes (36). AP is the only known ectonucleotidases which can fully degrade ATP and ADP into adenosine (9), and this was confirmed by our data that adenosine was generated as their turnover product. Platelet activation is known to be inhibited by the binding of adenosine to the platelet A2A (13) and A2B adenosine receptor (27). The A2A receptor is present in higher density on platelets, whereas the importance of the A2B receptor is limited to situations in which the A2B receptor density is strongly up-regulated or when pathophysiological adenosine concentrations are present (27). This could explain why the A2B antagonist PSB-1115 had no effect on recAP mediated platelet-inhibition in our experiments.

New pharmacological treatment options are urgently needed in sepsis. In this study, recAP was administered preceding ADP-induced platelet activation, thereby mimicking a prophylactic setting. Whether recAP will also exert protective effects in a more clinically relevant setting, is currently investigated in a large multicenter randomized controlled phase II trial in patients with sepsis-associated AKI. Next to the dephosphorylation of endotoxin and ATP, prevention of excessive platelet activation may also contribute to the beneficial effects of recAP in sepsis and sepsis-associated AKI as platelets are highly activated during sepsis (37). Increased platelet aggregation, platelet-endothelium binding, platelet-leukocyte complex formation and accelerated fibrin formation all contribute to renal microvascular thrombosis (38). Platelet-derived P-selectin was identified as a key component in this process (39) and it is well conceivable that this cascade is modulated by the observed effects of recAP as demonstrated by our observation that *ex vivo* recAP treatment reversed platelet hyperreactivity in sepsis patients. These results are in accordance with previous findings of Beumer and colleagues which showed that calf intestinal AP significantly reduced thrombocytopenia in piglets intravenously injected with LPS (40). *In vitro* exposure of platelets to LPS does not lead to strong platelet activation (41, 42) and we therefore refrained from performing experiments using LPS in the present study. It is therefore of interest to investigate the effect of recAP on platelet activation in sepsis patients *in vivo*.

Several studies reported that sepsis resulted in platelet hyporeactivity (43-45), a finding dissimilar to that of ours and others (19, 46-48). The seemingly conflicting data of platelet activation in sepsis may be explained by several things. Firstly, we used a flow-cytometry based assay to measure platelet function. Most studies reporting platelet hyporesponsiveness used light transmission aggregometry in PRP, which are less reliable when platelet counts are low (49), as frequently occurring in sepsis. Moreover, as described

by Woth and colleagues, it is difficult to detect platelet activation with secondary platelet exhaustion using this aforementioned technique (45).The use of PRP when compared with whole blood flow cytometry is also a disadvantage as mechanical manipulation may cause artifactual pre-activation of platelets (49). Additionally, in the *in vivo* setting of sepsis and in that of ours, activated leukocytes and endothelial cells in the milieu of whole blood may interact with and influence the platelet microenvironment, in contrast to when PRP is used. Leukocytes possess ATP-ase which may convert ATP present in whole blood into ADP, and in turn contribute to platelet hyperreactivity (50, 51).

The ability of AP to inhibit platelet activation, as measured by light transmission aggregometry, was first shown more than 20 years ago using bovine-derived kidney AP (14-16). Since then, no new data on the platelet effects of AP have emerged. Our current data add to the existing knowledge that the novel human recombinant product has similar platelet-inhibiting effects and that the dephosphorylation of ADP and generation of adenosine account for these effects. In the earlier studies, modification of platelet membrane receptors was speculated to mediate the platelet effects (15, 16), but our current experiments do not lend support for this mechanism.

As reviewed recently by Thomas and Storey (52), increasing data on the effects of platelet inhibitors on inflammation are available. However, these data are not easy to interpret as some studies have shown benefit of the P2Y12 inhibitors in sepsis patients (53-55), whereas others did not (55-58). Regardless, the inhibition of platelet reactivity to ADP reduces the release of pro-inflammatory mediators from platelet α-granules (52, 59). In addition, Ticagrelor, a P2Y12 receptor ADP antagonist, shows additional anti-inflammatory effects through a secondary inhibition of adenosine reuptake (60). Recently, our group showed that platelets modulate the cytokine responses of monocytes, and that ticagrelor administration in healthy volunteers reversed these effects partly through the inhibition of platelet-monocyte interaction (41). These immune-modulating effects of platelets could explain the observation that bovine-intestinal alkaline phosphatase administration reduced systemic levels of several inflammatory mediators during sepsis-associated AKI (5, 6).

In conclusion, we demonstrated for the first time that recAP inhibits *ex vivo* human platelet activation through the dephosphorylation of ADP, an important platelet agonist, and the formation of adenosine as its turnover product. This platelet-inhibiting effect may, at least partly, account for the therapeutic potential of recAP as a new candidate to treat sepsis-induced AKI. In addition, these results may pave the way to further investigate the effects of recAP treatment in other diseases in which platelet-activation plays a relevant role.

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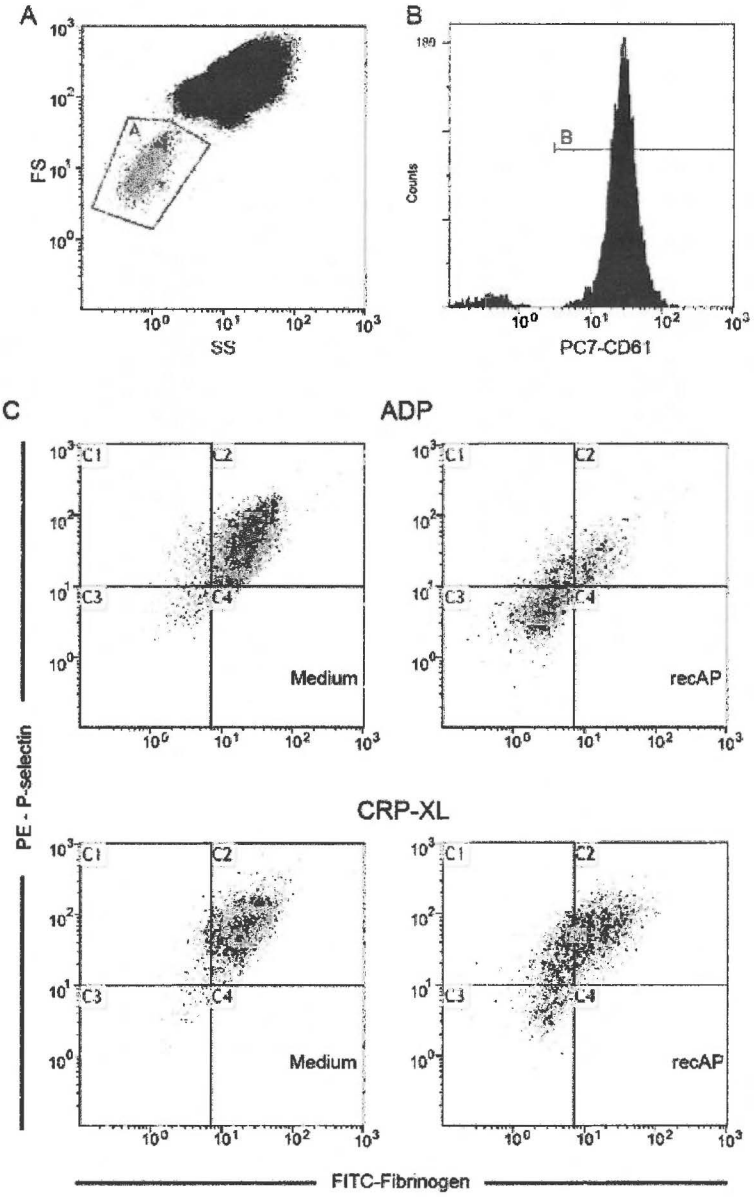
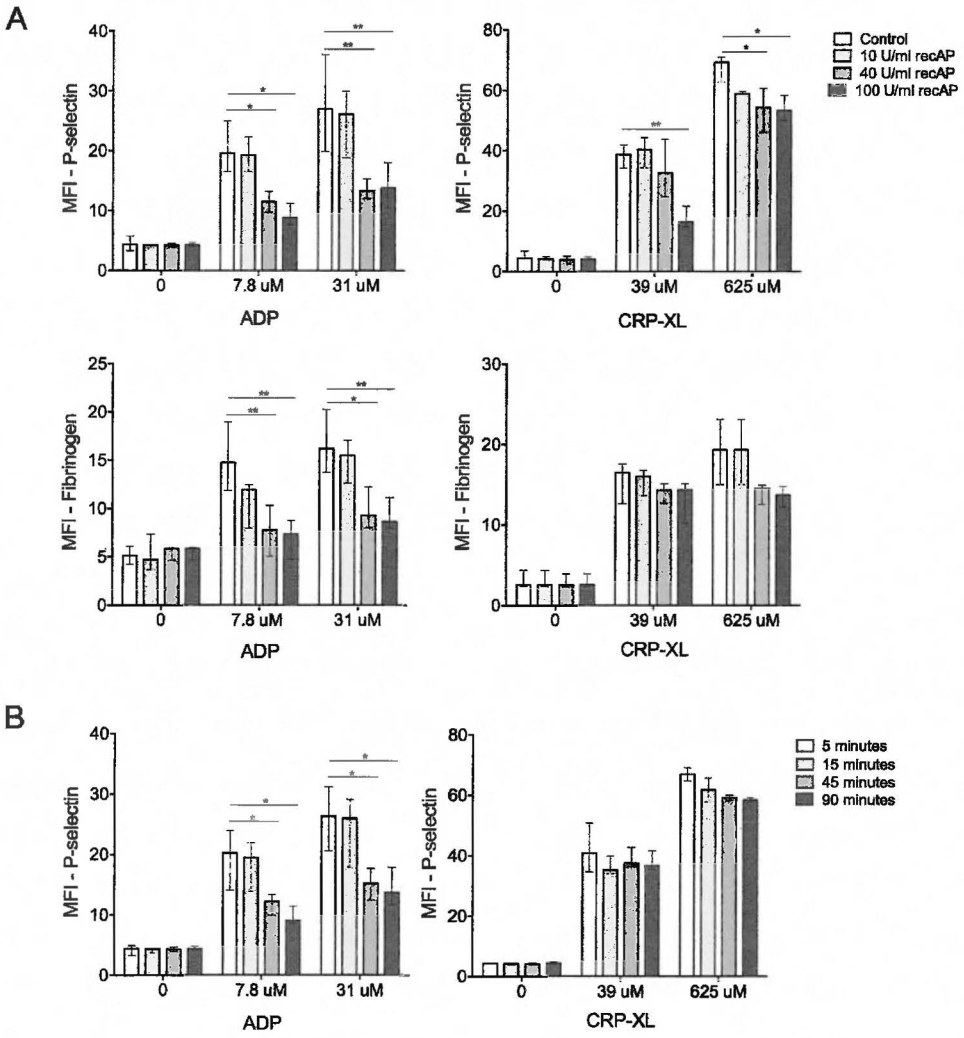
Supplemental Figures

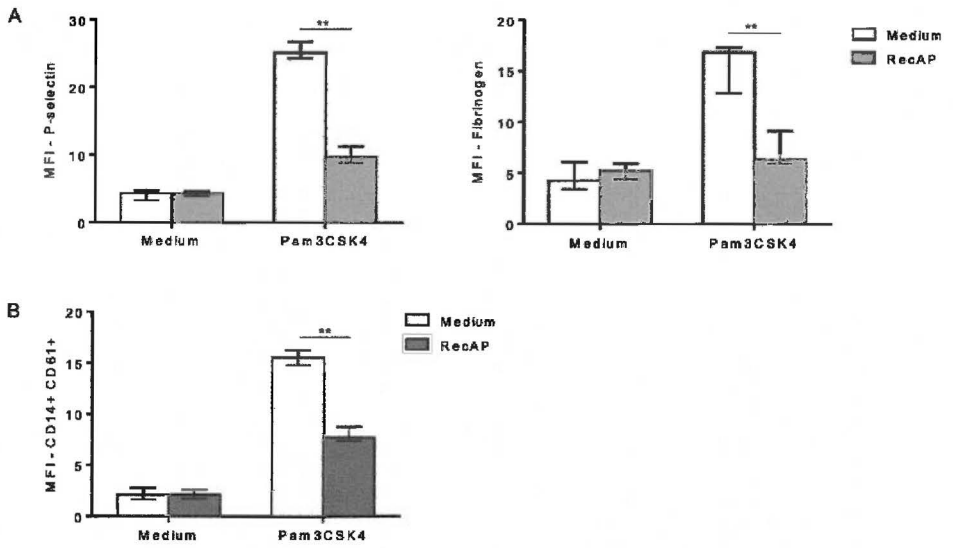


Supplemental Figure 1. Flow cytometry gating strategy for determination of P-selectin expression on platelets and platelet-fibrinogen binding.
Platelets are gated based on forward and side scatter characteristics (A) followed by positivity for the platelet marker CD61 (B). The median fluorescence intensity (MFI) of P-selectin and fibrinogen, after stimulation with ADP (31 μM) and CRP-XL (625 μM), was determined from the gated platelet population (C).



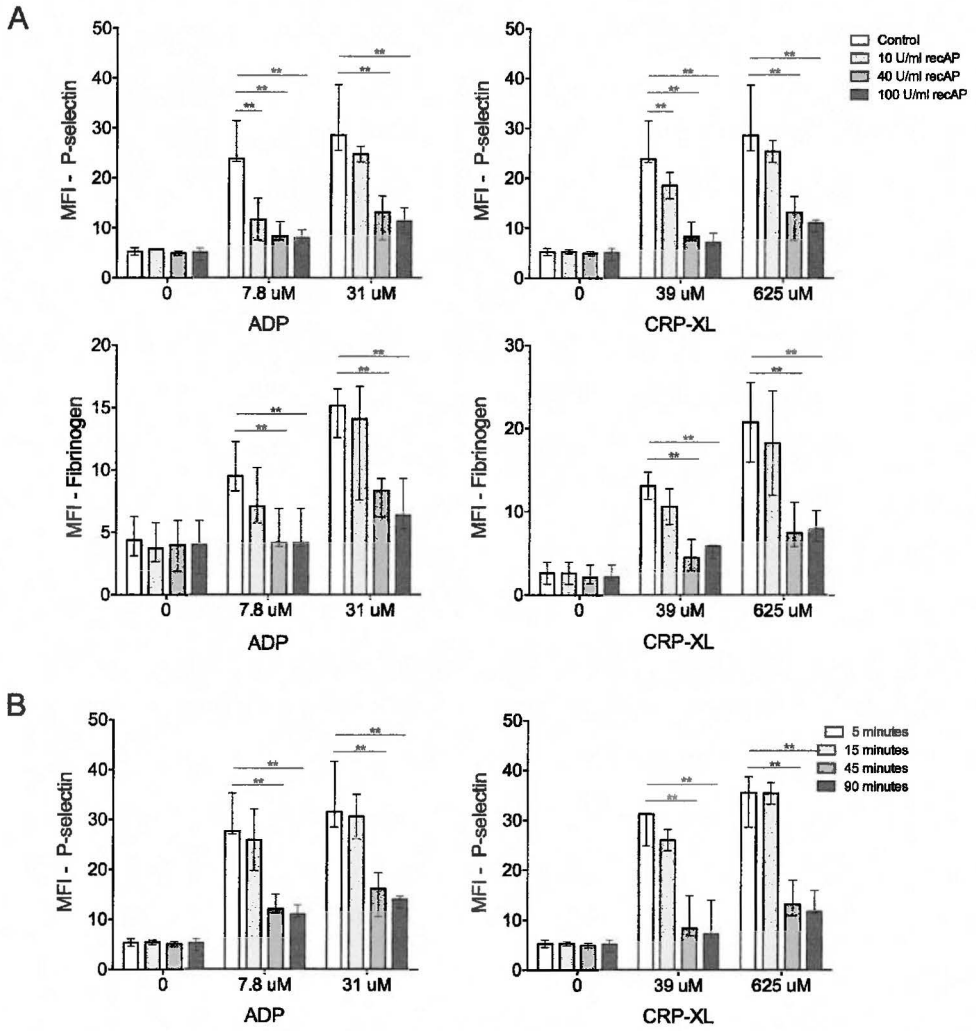
Supplemental Figure 2. The platelet-inhibiting effect of recAP in whole blood is dose- and time-dependent.

(A) Whole blood from healthy volunteers was pre-treated with different concentrations of recAP for 45 minutes prior to stimulation using low and high concentrations of ADP or CRP-XL. (B) Whole blood from healthy volunteers was pre-treated with recAP (40 U/ml) with different incubation times prior to stimulation using ADP and CRP-XL. The median fluorescence intensity (MFI) of the platelet surface expression of P-selectin and platelet-fibrinogen binding was measured by using flow cytometry. Data are presented as medians with IQR from 6 healthy donors. * $P < 0.05$, ** $P < 0.01$.



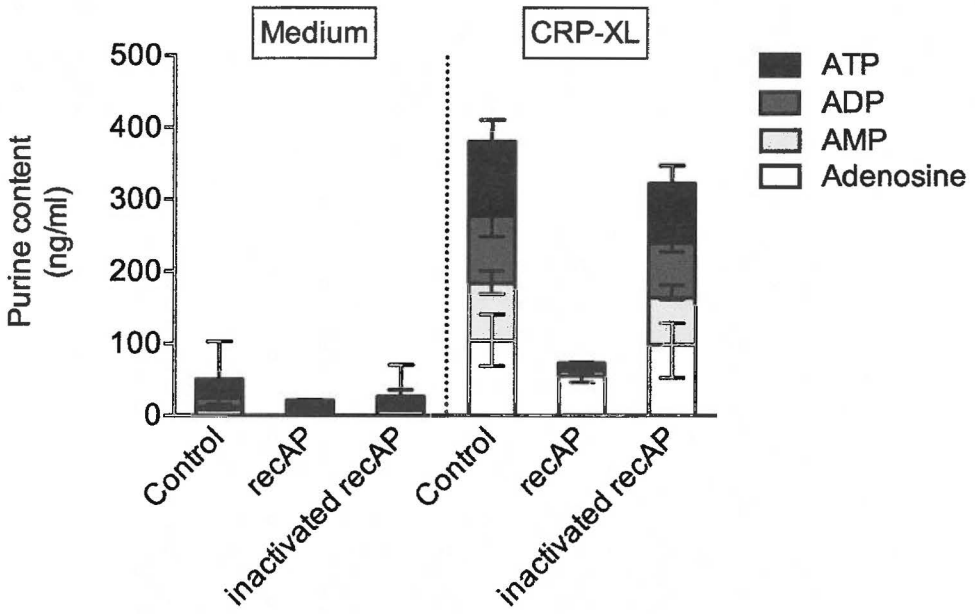
Supplemental Figure 3. The platelet-inhibiting effect of recAP on Pam3CSK4-stimulated whole blood.

(A) Whole blood was pre-treated for 45 minutes with medium or recAP (40 U/ml) prior to stimulation with Pam3CSK4. (B) Platelet-monocyte complex formation after exposure to Pam3CSK4 was determined by quantifying the MFI of the platelet marker CD61 on CD14+ cells. Data are presented as medians with IQR from 6 healthy donors. ** $P < 0.01$.



Supplemental Figure 4. The direct platelet-inhibiting effect of recAP in platelet-rich plasma (PRP) is dose- and time-dependent.

(A) PRP from healthy volunteers was pre-treated with different concentrations of recAP for 45 minutes prior to stimulation using low and high concentrations of ADP or CRP-XL. (B) PRP from healthy volunteers was pre-treated with recAP (40 U/ml) with different incubation times prior to stimulation using ADP and CRP-XL. The median fluorescence intensity (MFI) of the platelet surface expression of P-selectin and platelet-fibrinogen binding was measured by using flow cytometry. Data are presented as medians with IQR from 6 healthy donors. * $P < 0.05$, ** $P < 0.01$.



Supplemental Figure 5. Measurement of purine content in CRP-XL-induced PRP.
Purine levels were determined in the supernatant of PRP pre-treated with recAP (40 U/ml) or inactive recAP (in equivalent protein content) and subsequently stimulated with CRP-XL (39 uM). Presented data are medians with IQR from 4 healthy donors.

Chapter 5

Effects of hypoxia on platelet function and coagulation during systemic inflammation in humans *in vivo*

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Submitted.

Abstract

Introduction: Systemic inflammation and hypoxia both have been associated with increased platelet activation and hypercoagulability. Patients with critical illness often suffer from concurrent inflammation and hypoxia, and the possibly synergistic effects on platelets and coagulation may affect organ function and outcome. However, human data concerning the interaction between inflammation and hypoxia on platelet function and coagulation is lacking.

Materials and methods: Systemic inflammation was elicited by intravenous administration of lipopolysaccharide (2 ng/kg) in 20 healthy male volunteers, who were randomized to concurrent normoxia or hypoxia (oxygen saturation 80-85%). Platelet counts, platelet monocyte complexes (PMCs), platelet reactivity, thrombin generation and plasma levels of platelet factor 4 (PF4), von Willebrand Factor (vWF), and thrombin-antithrombin (TAT)-complexes were determined.

Results: Systemic inflammation resulted in a decrease in platelet counts (13% from baseline), and an 240% increase in PMCs and 35% increase in platelet reactivity. These endotoxemia-induced effects were attenuated by concurrent hypoxia. Systemic inflammation also increased plasma levels of vWF and TAT-complexes by 330 and 60% respectively, but did not affect plasma levels of PF4 and thrombin generation. Hypoxia neither modulated plasma concentrations of vWF, TAT-complexes, and PF4, nor thrombin generation.

Conclusion: Systemic hypoxia during systemic inflammation does not augment, but rather attenuates endotoxemia-induced decreases in platelet counts and endotoxemia-induced PMC formation and platelet reactivity. Therefore, our study provides no evidence for procoagulant effects of hypoxia in vivo in humans.

Keywords

inflammation, hypoxia, platelet, coagulation, endotoxin, thrombin

Introduction

Systemic inflammation in critical illness, as observed in patients with sepsis and following trauma, results in the activation of platelets and coagulation, which can ultimately lead to coagulopathy, organ dysfunction and worse outcomes (1,2). Concurrently, systemic hypoxia is common in critically ill patients, and is independently associated with adverse outcome (3), and tissue hypoxia may arise during inflammatory conditions as a result of enhanced metabolic demands, and decreased oxygen delivery(4). Hypoxia has also been implied to activate coagulation, as it has been associated with an increased risk of thrombotic events(5). Furthermore, hypoxia has been shown to increase platelet reactivity in rats(6) and enhance the procoagulant activity of human endothelial cells *in vitro*(7). Therefore, hypoxia may also contribute to altered platelet function and coagulopathy in critically ill patients, increasing their risks for organ dysfunction. However, the limited number of human studies available have mainly focused on the effects of hypoxia on plasmatic coagulation, and most of them do not support procoagulant effects of hypoxia(8,9). Human *in vivo* studies on the effects of hypoxia on platelet function did also not identify any effects, but this was only investigated during very mild (10) or using very short bouts(11) of hypoxia.
Although hypoxia and inflammation may both affect platelet function and plasmatic coagulation, human *in vivo* data on the interaction between hypoxia and inflammation on coagulation are completely lacking. We recently reported that systemic hypoxia exerts profound anti-inflammatory effects in humans *in vivo* during experimental human endotoxemia (Kiers D. et. al., 2016, submitted). In the present work, we describe the effects of systemic hypoxia during human endotoxemia on platelet function, endothelial cell activation and plasmatic coagulation.

Methods

Study design and population:
Data were obtained from a previously reported parallel randomized controlled experimental endotoxemia study (Kiers D. et. al., 2016, submitted). In this study subjects were randomly assigned to normoxia (room air) or hypoxia (peripheral oxygen saturation (SaO_2 80-85%). All study procedures were in accordance with the declaration of Helsinki, including the latest revisions. The study was performed after approval of the local ethics committee. Healthy, non-smoking, male volunteers aged 18-35 years were included after written informed consent and a screening with a normal physical examination, routine laboratory tests and ECG. Exclusion criteria were use of prescription medication and recent visits to high altitude or airplane flights.

Experimental protocol

The endotoxemia experiments were performed as previously described (Kiers D. et. al., 2016, submitted). Briefly, purified lipopolysaccharide (LPS, US Standard *Escherichia coli* O:113 endotoxin, National Institute of Health, Bethesda, MD, USA) was administered as an i.v. bolus of 2 ng/kg. All subjects received infusion of 1.5L glucose 2.5%/ NaCl 0.45% in one hour before LPS administration, and were fitted with an air-tight respiratory helmet (StarMed, CaStar, Italy). Room air was supplied to the helmet of the subjects randomized to normoxia. In the hypoxia group, a gas mixture of nitrogen and room air was titrated to achieve a SaO_2 of 80-85%. Subjects wore the respiratory helmet for a period of 3.5 h, starting one hour before LPS administration. Blood was collected in 3.2% citrate anti-coagulated vacutainers.

Platelet counts, platelet monocyte complexes and platelet reactivity

Platelet counts were determined using an automated hematology cell counter. Platelet monocyte complexes (PMCs) were measured by incubating whole blood with fluorescent antibodies against CD14 as monocyte identification and CD42b as a platelet marker. PMCs were identified with flow cytometry as monocytes positive for the platelet marker. Platelet reactivity was measured with flow cytometry as described previously (12). Briefly, platelet reactivity was measured by staining whole blood with PE-labeled anti-CD62P (Bio-Legend, San Diego, USA) and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). Samples were incubated with eight concentrations of adenosine diphosphate (ADP; Sigma Aldrich, Germany). The mean fluorescent intensity (MFI) of CD62P on platelets was determined for each ADP concentration with flow cytometry. The area under the ADP-response curve was used as a measure for platelet reactivity.

Calibrated automated thrombin generation

Thrombin generation was measured by means of calibrated automated thrombography as described by Hemker et al.(13) Briefly, platelet poor plasma and a calibration sample with known thrombin activity were incubated with a mixture of tissue factor and phospholipids to induce thrombin generation. Hereafter, a fluorogenic, calciumchloride and BSA was added. Fluorescence, as a measure of thrombin conversion, was measured for one hour on a microplate reader. The endogenous thrombin potential (ETP) is the total amount of thrombin formed in one hour and is calculated by comparing the fluorescent signal of the subject sample with calibrator sample.

ELISAs

Plasma concentrations of von Willebrand factor (vWF), platelet factor-4 (PF4) and thrombin-antithrombin (TAT) complexes were measured using ELISA's, as previously described (14).

Statistical analysis

The effect of endotoxemia was evaluated by performing a one-way analysis of variance on the data of normoxic subjects. To evaluate the effects of hypoxia versus normoxia during endotoxemia, general linear mixed models were used. Data were analyzed with SPSS version 22 (IBM, Chicago, USA). A p-value of <0.05 was considered significant.

Results

Platelet counts, platelet monocyte complexes, platelet reactivity and platelet factor 4 levels
The experimental procedures, including hydration with 1.5 L of fluid and induction of a systemic inflammatory response induced a slight, but significant, decrease in platelet counts in the normoxic group ($p<0.0001$), which was less pronounced in the hypoxia group (Fig 1A). In the normoxic subjects, the percentage of PMCs increased during endotoxemia ($p=0.0006$, Fig 1B). Hypoxia attenuated the formation of PMCs. Platelet reactivity showed a very similar pattern as PMCs, peaking at 2.5h after LPS administration in normoxic subjects ($p<0.0001$, Fig 1C). Hypoxia blunted the endotoxemia-induced increase in platelet reactivity. Plasma levels of PF4 in normoxic subjects tended to increase in the first 3 hrs following endotoxemia ($p=0.08$, Fig 1D). Hypoxia did not modulate this response.

Von Willebrand factor levels and plasmatic coagulation
Systemic inflammation resulted in increased plasma concentrations of vWF ($p<0.0001$), with a trend towards slightly higher levels in the hypoxia group that did not reach statistical significance ($p=0.06$, Fig 1E). Plasmatic coagulation was evaluated by measuring thrombin generation and TAT complexes. Endotoxemia tended to induce a short-lived increase in thrombin generation ($p=0.08$, Fig 1F), and resulted in increased concentrations of TAT complexes ($p=0.03$, Fig 1G). Both measures of plasmatic coagulation were unaffected by hypoxia.

Discussion

Herein, we report that systemic hypoxia attenuates endotoxemia-induced effects on platelets. Endotoxemia-induced decreases in platelet counts as well as increases in PMCs and platelet reactivity were all mitigated by concurrent hypoxia. Furthermore, we demonstrate that hypoxia does not modulate endotoxemia-induced increase in plasma levels of vWF and TAT-complexes. The effects of endotoxemia on platelet degranulation marker PF4 and thrombin generation, as a measure of plasmatic coagulation, were not modulated by hypoxia.
In line with our results, previous reports have consistently identified that human endotoxemia results in decreased platelet counts(15) as well as increased PMCs(16), platelet reactivity(17), plasma levels of vWF(15,17) and TAT complexes(18). Correspondingly, observational studies in patients with sepsis have also identified increases in markers of platelet activation(19). To the best of our knowledge, thrombin generation has not been previously assessed during endotoxemia and we only observed

a borderline significant short-lived rise in thrombin generation potential that was not affected by hypoxia.

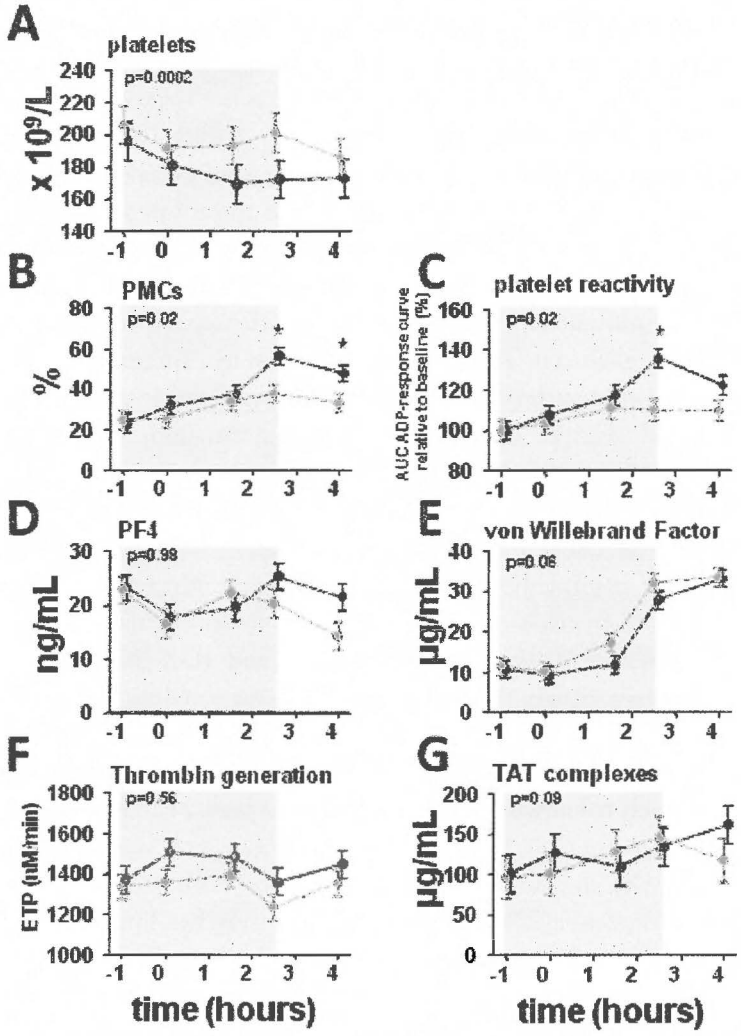

Figure 1. Time course of platelet function and coagulation parameters during experimental endotoxemia in normoxic and hypoxic healthy volunteers.

(A) Whole blood platelet counts. (B) Percentage of platelet monocyte complexes (PMCs). (C) Platelet reactivity expressed as the change from baseline of the area under the adenosine diphosphate dose-response curve. (D) Plasma concentrations of platelet factor 4 (PF4). (E) Plasma concentrations of von Willebrand Factor. (F) Endogenous thrombin generation. (G) Plasma concentrations of thrombin-antithrombin (TAT) complexes. The period that subjects were hypoxic or normoxic is indicated with a grey box. LPS was administered at t=0 hours. Data are expressed as the estimated mean with error obtained from the mixed linear model. P-values express the difference between the normoxic and hypoxic endotoxemia model.

The few human studies that investigated the influence of hypoxia on platelets reported no effects of either eight hours of mild hypoxia or a seven minute period of deep hypoxia(11) on soluble P-selectin levels, platelet-monocyte complexes and platelet reactivity. In contrast, severely hypoxic rats exhibited increased platelet activation and aggregation(13). Previous studies on the effect of hypoxia on plasmatic coagulation have shown conflicting results. One reported that acute hypoxia results in increased concentrations of prothrombin fragments 1+2 (F_{1+2}) and TAT complexes(8), both markers of thrombin formation. In contrast, other studies have shown no effects of hypoxia on either $F_{1+2,}$ TAT complexes, endogenous thrombin generating potential or several other measures of plasmatic coagulation(16–18). Taken together, most studies on the effects of hypoxia in humans *in vivo* do not identify procoagulant effects, which is consistent with our current findings. Therefore, the assumption that hypoxia itself results in a procoagulant state *in vivo* is not supported by experimental data.

Several potential mechanisms may explain the attenuating effects of hypoxia on endotoxin-induced changes in platelet counts and function. LPS is the archetypal ligand for Toll Like Receptor (TLR)-4, and although platelets express TLR4, the ability of LPS to directly activate platelets is debated(19). Therefore, it is plausible that the endotoxemia-induced effects on platelets are mediated by an indirect effect of endotoxin administration, for example by the LPS-induced release of cytokines. Because subjects that were exposed to hypoxia exhibited 40 to 50% lower circulating levels of the pro-inflammatory cytokines TNFα, interleukin(IL)-6 and IL-8 (Kiers D. et. al., 2016, submitted). This may explain the attenuated effects on platelets. Alternatively, hypoxia might have resulted in increased levels of prostaglandin I2 (PGI_2), which is known to inhibit platelet activation(20). Lastly, it has been reported that deoxygenated erythrocytes produce NO, which is known to inhibit platelets as well(21). Reliable measurements of PGI_2 production can only be performed in urine, which was not collected in our study. Plasma nitrite measurements, as a proxy for NO, are not increased during experimental endotoxemia(22). Furthermore, our study has limitations related to the experimental human endotoxemia model used, which naturally does not fully represent the inflammatory response observed in critically ill sepsis patients. Nevertheless, given the paucity of human *in vivo* data on the subject, it provides valuable insights into the complex interactions between inflammation, coagulation, and hypoxia.

Conclusion

Systemic inflammation and hypoxia have been associated with enhanced platelet activity and hypercoagulation, both of which may contribute to organ dysfunction and affect outcome in critically ill patients. We report that systemic hypoxia during systemic inflammation in humans *in vivo* does not augment, but rather attenuates, endotoxemia-induced decreases in platelet counts and endotoxemia-induced increased PMC formation and platelet reactivity.

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Chapter 6

Invasive pneumococcal disease leads to activation and hyper-reactivity of platelets

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Abstract

Using a novel porcine model of intravenous *Streptococcus pneumoniae* infection, we showed that invasive pneumococcal infections induce marked platelet activation and hyperreactivity. This may contribute to the vascular complications seen in pneumococcal infection.

Keywords: platelets, pneumonia, *Streptococcus pneumoniae*, myocardial infarction, platelet activation, infection

Introduction

Community acquired pneumonia (CAP) is associated with an increased short-term and long-term risk for cardiovascular events (CVE) (1). The Gram-positive bacterium *Streptococcus pneumoniae* is a major cause of CAP and sepsis. *S. pneumoniae* is well known to interact with and activate platelets *ex vivo* (2). Platelets play a central role in acute CVE and atherosclerosis (3), and we speculate that excessive platelet activation may also contribute to the vascular comorbidity in pneumococcal infections. However, whether systemic platelet activation and platelet hyperreactivity are prominent features of invasive pneumococcal infections *in vivo* is not well-established.
Porcine animal models are frequently used in cardiovascular research due to the similarities with humans in terms of cardiac anatomy and the haemostatic system (4). Pigs are also a natural host of the pathogen *Streptococcus suis*, which has strong genetic similarities with *S. pneumoniae*. Using a novel porcine model of pneumococcal disease, we investigated the hypothesis that invasive pneumococcal infections are associated with pronounced platelet activation and hyperreactivity.

Methods

Porcine S. pneumoniae infection model
Full details of the animal experimental model are described elsewhere (5). In short, two groups of five piglets received 1 ml intravenous injection of either a low ($4.2x10^6$ Colony-Forming Unit [CFU]/ml) or high dose ($2.9x10^8$ CFU/ml) of *S. pneumoniae* strain PBCN214, a serotype 8 invasive strain isolated from a patient with pneumonia, meningitis and sepsis. Two other groups of five piglets each received 3 ml of intranasal inoculation of a low ($2.5x10^6$ CFU/ml) or high dose ($2.9x10^8$ CFU/ml) of *S. pneumoniae* strain BHN418, a serotype 6B strain previously used in experimental human carriage models (6). Piglets were euthanized at day seven post-inoculation, or earlier when reaching pre-defined humane end points. The animal experiment was approved by the Ethical Committee of the Central Veterinary Institute of Wageningen UR (The Netherlands), in accordance with the Dutch law on animal experiments (permit number 2014004b).

Porcine platelet activation and reactivity assay
Platelet activation and reactivity were measured using a flow cytometry-based assay, as previously described for pigs (4). The membrane expression of the platelet alpha-granule protein P-selectin and the binding of fibrinogen to the activated αIIbβ3 receptor were determined using KO2.5-Alexa Fluor 488 (AbD Serotec, Oxford, UK) and F0111-

FITC (DAKO Ltd., High Wycombe, UK) antibodies in unstimulated, citrated whole blood and after *ex vivo* stimulation of whole blood by two concentrations (7.8 and 31.2 mmol/L) of adenosine diphosphate (ADP).

In vitro activation of porcine and human platelets by S. pneumoniae
The ability of the used *S. pneumoniae* strains to directly activate porcine and human platelets was investigated by incubating whole blood and isolated washed platelets with $1x10^8$ CFU/mL of heat-killed *S. pneumoniae* (strains PBCN214 and BHN418) for 1hr at 37°C. The Gram-negative bacterium *Escherichia coli* was included as a control. Samples were fixed and platelet P-selectin expression and platelet fibrinogen binding were measured using flow cytometry as described above.

Statistical analysis
Data are expressed as arithmetic means with standard error of the means (SEM). Daily changes in platelet reactivity were compared to baseline pre-infection values using a linear mixed model with post hoc Bonferroni-Holm correction. Differences in the *in vitro* experiments were analyzed with standard statistical tests. $P<0.05$ was considered statistically significant.

Results

All piglets in the intravenous (IV) groups developed fever and positive blood cultures for *S. pneumoniae*, whereas all piglets in the high dose intranasal (IN) group were colonized by *S. pneumoniae* throughout the duration of the experiment. One piglet in the latter group had a transient bacteremia. Four of five animals in the high dose IV group and one animal in the low dose IV group developed arthritis, which necessitated premature euthanization in two animals from the high dose IV group (at day 2 and 4, respectively). Detailed clinical and histological findings of the experimental model is described elsewhere (5).
Piglets in the high dose IV group, but not those in the low IV group or the intranasal groups, demonstrated a transient decrease in mean (SEM) circulating platelet count from $489\pm58x10^9$/L at baseline to $353\pm37x10^9$/L ($P=0.016$; paired T-test) at day one after intravenous inoculation (Fig. 1A). This was associated with a significant increase in platelet CD62P expression and fibrinogen binding in both unstimulated samples and upon *ex vivo* stimulation with a low and high dose of ADP (representative flow-cytometry plots shown in Fig. 1B, platelet reactivity data shown in in Fig 1C). There was a weak correlation of CD62P expression in unstimulated samples at day one and the relative decrease in platelet number between baseline and day one in the high dose IV

group (Spearman R=0.66; *P*=0.04). No change in platelet activation markers was found in piglets in both IN groups.

Next, we determined the ability of the used *S. pneumoniae* strains to directly activate porcine and human platelets *in vitro*. The Gram-negative bacterium *Escherichia coli* was included as control. Whereas *E. coli* did not upregulate platelet P-selectin expression and fibrinogen binding, both strains of *S. pneumoniae* induced the expression of these platelet activation markers (Fig. 1D), suggesting that the observed platelet hyperreactivity in the experimental model above was not merely a result of inflammation, but also through direct platelet stimulation by *S. pneumoniae*.

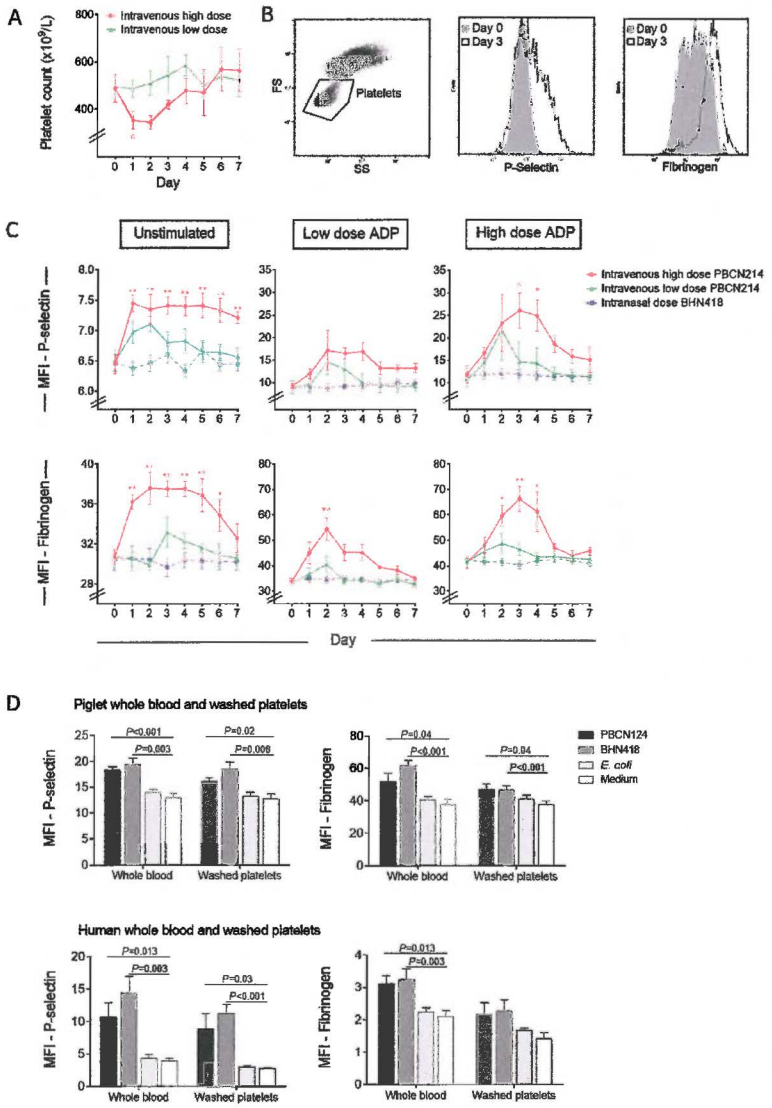

Fig 1.

(A) Time course of platelet count in piglets (n=5 per group) inoculated intravenously with 1 ml of low ($4x10^6$ CFU/mL) or high ($2.9x10^8$ CFU/mL) dose of the invasive *S. pneumoniae* strain PBCN214. **P*<0.05 versus the baseline sample before inoculation (paired T-test). **(B)** Representative plots showing the platelet gating strategy whereby platelets were selected on their forward and side scatter characteristics (left panel), and observed differences in platelet P-selectin expression (middle panel) and platelet fibrinogen binding (right panel) after *ex vivo* stimulation with 31.2 mmol/L ADP between pre-infection sample (day 0) and day 3. **(C)** Time course of P-selectin expression and fibrinogen binding, expressed as mean fluorescence intensity (MFI) in arbitrary units, in unstimulated samples and after *ex vivo* stimulation by 7.8 or 31.2 mmol/L ADP. **P*<0.05, ***P*<0.01 versus the baseline sample before inoculation (linear mixed models). **(D)** P-selectin expression and fibrinogen binding of porcine and human washed platelets and whole blood following *ex vivo* incubation with the invasive (PBCN214) and colonizing (BHN418) strains of *S. pneumoniae* and by *Escherichia coli* (both $1x10^8$ CFU/ml). Data presented are aggregated from a total of 6 piglets and 9 humans. *P* values determined using T-tests. Data in this figure are presented as means with standard error of the means (SEM).

Discussion

This study demonstrates that invasive *S. pneumoniae* infections induce pronounced platelet activation and hyperreactivity. Direct stimulation of platelets by *S. pneumoniae* mediated these effects, at least in part, as porcine washed platelets were activated by *ex vivo* exposure to *S. pneumoniae*.

Pneumococcal pneumonia is an independent risk factor for acute CVE among patients with CAP (7). Platelets are key cells in acute CVE and the observed platelet hyperreactivity may contribute to the increased risk for vascular thrombosis in patients with pneumococcal infections. Our data are consistent with a patient study showing increased plasma markers of platelet activation in CAP patients with signs of myocardial infarction (3).

Pneumococci can activate platelets directly through different processes, including binding to platelet Toll-like receptor (TLR)-2, FcγRIIA and the integrin αIIbβ3 (2). In addition, phosphorylcholine residues of the pneumococcal cell wall may also serve as a platelet activation factor (PAF) mimetic as well as a bacterial adhesin to PAF receptors, which has been reported to be present on porcine platelets (8). Finally, piglets in the intravenous group developed fever and significant increases in the pro-inflammatory cytokines interleukin(IL)-1β and IL-6 (5). Inflammation and the resulting activation of the endothelium and plasmatic coagulation may also contribute to platelet activation.

Constraints of our study include the fact that a large animal model is restricted in the number of animals used, especially because two of the piglets in the high dose IV group were euthanized before the end of the study. The group of intranasally inoculated piglets function as a control group showing the stability of our platelet assay in time. Secondly, the used model did not allow us to relate platelet hyperreactivity to CVE. Thirdly, the invasive *S. pneumoniae* strain was intravenously inoculated. Although pneumococcal bacteremia is a common complication of pneumococcal pneumonia, our findings cannot directly be translated to pneumococcal pneumonia without bacteremia.

ADP is known for its role in the amplification of platelet activation by other platelet agonists, and was the sole platelet agonist used in this study. Other commonly used platelet agonists in humans, such as thrombin receptor activating peptides (TRAP) and collagen, are known to incompletely activate porcine platelets (4).

The findings of the epidemiologic studies mentioned above, together with our current findings, strengthen the case for platelet function inhibitors as a preventive intervention to reduce the number of acute vascular thromboses during invasive pneumococcal infections. A small placebo controlled trial involving 185 patients found that aspirin reduced the occurrence of CVE in CAP patients from 10.6% to 1.1% (9). In addition, a large observational study involving over 1000 elderly CAP patients reported lower 30-day mortality rate for those who had been on chronic treatment with aspirin, compared

to those who had not (10). Larger placebo-controlled studies are warranted before evidence-based recommendations can be given. A pneumococcal porcine model as used in our study may allow for identification of platelet inhibitors to prevent pneumococcal-induced platelet activation.

In conclusion, invasive pneumococcal infections induce pronounced *in vivo* platelet activation and platelet hyperreactivity and this may contribute to the increased incidence of vascular complications seen in these infections.

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Chapter 7

Phage-derived protein induces increased platelet activation and is associated with mortality in patients with invasive pneumococcal disease

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Abstract

To improve our understanding about the severity of invasive pneumococcal disease (IPD), we investigated the association between the genotype of *Streptococcus pneumoniae* and disease outcome of 349 bacteremic patients. Pneumococcal genome-wide association (GWAS) analysis demonstrated a strong correlation between 30-day mortality and the presence of the phage-derived gene, *pblB*, encoding a platelet-binding protein of which effects on platelet activation were previously unknown. Platelets are increasingly recognized as key players of the innate immune system, and in sepsis, excessive platelet activation contributes to microvascular obstruction, tissue hypoperfusion and finally multi-organ failure leading to mortality. Our *in vitro* studies revealed that *pblB* expression was induced by fluoroquinolones but not by the beta-lactam antibiotic Penicillin G. Subsequently, we determined *pblB* induction and platelet activation by incubating whole blood with wild type or knock-out mutant of *pblB*, in the presence or absence of antibiotics commonly administered in our patient cohort. *PblB*-dependent enhancement of platelet activation, as measured by increased expression of the α-granule protein P-selectin, the binding of fibrinogen to the activated αIIbβ3 receptor and the formation of platelet-monocyte complex occurred irrespective of the antibiotics exposure. In conclusion, the presence of *pblB* on the pneumococcal chromosome potentially leads to increased mortality in patients with an invasive *S. pneumoniae* infection, which may be explained by enhanced platelet activation. This study highlights the clinical utility of bacterial GWAS, followed by functional characterization to identify bacterial factors involved in disease severity.

Keywords: *Streptococcus pneumoniae*, mortality, *pblB*, fluoroquinolones, platelet activation

Introduction

Streptococcus pneumoniae or the pneumococcus is a frequent colonizer of the nasopharynx. In a minority of carriers, infection progresses to pneumococcal disease with an estimated 1.6 million deaths annually (1, 2). The largest clinical burden of invasive pneumococcal disease (IPD) is seen in young children and older adults, who present mostly with sepsis and meningitis. Case mortality rates are estimated to range from 11 to 30% in adults (3-5), with treatment becoming complicated due to the worldwide emergence of multi-drug resistance (6). Therefore, it is of utmost importance to fully understand the pathogenic mechanisms of pneumococcal disease in order to improve treatment and prognosis of critically ill patients.

Recently, the utilization of whole genome sequencing and analyses for predicting and understanding pathogen virulence has been highlighted (7). In this study, we performed a genome-wide association analysis (GWAS) on 349 pneumococcal draft genomes of blood isolates from patients who were admitted with IPD in two Dutch hospitals. We identified a significant association between 30-day mortality and the presence of *pblB*, encoding for a platelet binding protein that was also reported to function in adhesion (8). In a subsequent functional study, we investigated the induction of the phage-derived *pblB* expression by fluoroquinolones in *S. pneumoniae*. Lastly, we simulated the *in vivo* conditions using an *ex vivo* whole blood assay demonstrating the importance of PblB in enhancing platelet activation.

Platelets are an important part of the innate immune system, and can interact with and be activated by *S. pneumoniae*. In sepsis, platelet activation and platelet-leukocyte complex formation contribute to microvascular obstruction, tissue hypoperfusion and finally multi-organ failure (9). The role of this phage-derived gene in the clinical outcome and severity of IPD patients and its consequences for platelet activation warrant further study.

Materials and Methods

Study population. Consecutive patients hospitalized with a bacteremic pneumococcal infection at two Dutch hospitals between 2001 and 2011 were included in the study. Detailed clinical data were obtained on patient characteristics, clinical severity, treatment and the course of disease. Corresponding blood culture isolates of *S. pneumoniae* were collected and serotyped as described before (10). For 349 of the isolated strains, sequencing, assembly of draft genomes and annotation was determined as previously described (36). This study was reviewed and approved by the Local Medical Ethical Committees. All adult patients and healthy volunteers involved in this study provided written informed consent.

Orthologous clustering and GWAS. Orthologous genes (OGs) from *S. pneumoniae* used in this study have previously been described by our group (36). Putative protein coding sequences were investigated using an "all-versus-all" protein BLAST (blastP), with a 10e-15 *e*-value cut-off and a BLOSUM90 substitution matrix. The results were subsequently clustered into clusters of orthologous groups using TribeMCL (36, 37), resulting into a total of 3021 orthologous genes (OGs), 1075 of which were conserved in all isolates in a single copy. The population (sub)structure (sequence clusters; SCs) used for population stratification in the study have also been previously characterized (36). Basing disease severity on mortality within the first 30 days of admission to the hospital, the pneumococcal isolates were categorized into three categories: derived from patients who died (n=37), from patients who survived (n=309), and from patients of whom the data was not captured (n=3). The Cochran-Mantel-Haenszel (CMH) association statistics was employed to test the associations between the presence or absence of pneumococcal OGs and 30-day mortality, conditional on the bacterial population substructure as proposed by Bayesian Analysis of Population Structure (BAPS) analysis (11). All associations were determined using PLINK (38). Candidate OGs were selected based on association test with $p < 0.05$ (Bonferroni adjusted for multiple testing). Visualization of the results were performed using ITOL (39).

Adjustment for covariates of mortality. Potentially interesting covariates of 30-day mortality were analyzed using binary logistic regression analysis by likelihood ratio based backward modeling, entering the pneumococcal OG plus identified possible covariates as explaining variables. Detailed statistical methods are described in Method S1.

Induction of *pblB* expression by antibiotics. Three isolates randomly selected from the group of deceased patients, containing the *pblB* gene, were selected: PBCN0103, PBCN0226 and PBCN0239. Different concentrations of mitomycin C, penicillin G, ciprofloxacin and levofloxacin (all purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands) were tested to determine the sub-lethal doses. The pneumococci were grown in THY medium to midlog (OD 0.3), then diluted to OD 0.1, supplemented with 0.132 µg/ml mitomycin C, 0.0125 µg/ml penicillin G, 0.533 µg/ml ciprofloxacin or 0.533 µg/ml levofloxacin, and grown for an additional two hours at 37°C with 5% CO_2. Subsequently, serial dilutions were incubated on blood agar plates (BD) and incubated overnight at 37°C with 5% CO_2. Experiments were performed in triplicate to determine the expression of *pblB*. Mitomycin C was included as positive control, as it was previously shown to induce *pblB* expression (40). After two hours of growth, pneumococci were harvested by centrifugation. The supernatant was discarded and a 2:1 volume of RNA protect (Qiagen, Hilden, Germany) was added to the pellet. RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Residual DNA was removed with a DNAse treatment using the Ambion Turbo DNA-free kit according to manufacturer's instructions (Ambion, Austin, Tx,

USA). The qRT-PCR was performed as previously described by DeBardeleben *et al.* (40) using the following primers: HBgyrAF: AATGAACGGGAACCCTTGGT, HBgyrAR: CCATCCCAACCGCGATAC, pblB_F: TACAGCTGTGAAAGCCTTGG, pblB_R: GATAGCCATCTGGATTCTCAGG.

***Construction of S. pneumoniae* strain PBCN0162Δ*pblB*.** A directed gene deletion mutant of *S. pneumoniae* strain PBCN0162 was generated by allelic exchange of the target gene (*pblB*) with a spectinomycin resistance cassette (obtained from pR412T7), using the megaprimer polymerase chain reaction method, this resulted in PBCN0162Δ*pblB*. Briefly, flanking regions of ~500 bp, containing less than 150 bp of the coding sequence of the target genes, were amplified by PCR, with chromosomal DNA as the template. For each flanking region, the primer closest to the target gene (extension plus _L2 or _R2) contained an additional sequence complementary to primer PBpR412_L or PBpR412_R. In a second PCR, the PCR products of the two flanking regions and the antibiotic resistance cassette were combined, leading to incorporation of the antibiotic resistance cassette between the two flanking regions of the target gene, as previously described by Burghout et al., 2007 (41). The primer sequences are provided in Table S2 in the supplemental material. Subsequently, the megaprimer PCR product was used for transformation of competent PBCN0162. Mutants, selected on blood agar plates containing spectinomycin, were assessed by colony PCR for recombination at the desired location on the chromosome. Chromosomal DNA was isolated from the mutants and used for transformation of competent strain PBCN0162. Gene inactivation was confirmed by quantitative real-time PCR gene expression analyses as described above (see '*Induction of pblB expression by antibiotics*').

***Ex vivo* (whole blood) assays.** Whole blood was obtained from healthy volunteers (n=6) after informed consent using 3.2% citrate-anticoagulated tubes (BD Vacutainer, Becton Dickinson, Plymouth, UK) and exposed to $1x10^7$ CFU/ml Δ*pblB* or wt pneumococci for 30 minutes at 37°C. Subsequently, either medium, penG (0,0125 ug/ml), CPX (0,533 ug/ml), or a combination of penG and CPX were added, and samples were incubated for 2 hrs at 37°C. RNA isolation and qRT-PCR was performed as described in the previous section. These whole blood samples were also collected for measurement of platelet activation and PMC by flow cytometry.

Measurement of platelet activation and platelet-monocyte (PMC) complex formation by flow cytometry. Platelet activation was measured by whole blood flow cytometry as previously described (42) by quantifying the platelet membrane expression of the α-granule protein P-selectin (CD62P) and the binding of fibrinogen to the activated αIIbβ3 receptor (GPIIbIIIa complex). The following antibodies were used to incubate samples from the whole blood *ex vivo* assay: PE-labelled anti-CD62P (Bio-Legend, San Diego, CA, USA), FITC-labelled anti-fibrinogen (F0111-FITC; DAKO Ltd., High Wycombe, UK) and PC7-labelled anti-CD61 (*platelet glycoprotein IIIa*,

Beckman Coulter, Miami, FL, USA), the latter as platelet identification marker. The percentage of CD62P and fibrinogen on CD61-positive events were determined. Formation of PMC was measured by incubating samples with PC7-labelled anti-CD61 and PE-labelled anti-CD14 (a-glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein; Bio-Legend). After 20 min incubation, Optilyse B (Beckman Coulter, Fullerton CA, USA) was added to lyse erythrocytes. PMC formation was determined by quantifying the MFI of CD14+ cells that were also positive for the platelet identification marker CD61. All samples were measured using a FC500 flow cytometer (Beckman Coulter).

Statistical Analyses. Results from independent experiments (involving n = 6 donors) were pooled, data are provided as means with 95% confidence interval unless otherwise stated. Generalized linear mixed model with post-hoc Bonferroni corrections were used to statistically analyze our experimental data. In the *in vitro* induction of *pblB* in culture medium, antibiotics were analyzed as a fixed effect on *pblB* expression, whereas inter-donor variation was analyzed as a random effect (random intercept). For the whole blood assay, the presence or absence of bacteria and the different antibiotics, as well as their interactions, were analyzed as fixed effects on platelet activation and the inter-donor variation were analyzed as a random effect (random intercept). All analyses were performed using SPSS version 20 (SPSS, Chicago IL, USA). The level of significance was set at $p<0.05$.

Results

***pblB* is an independent determinant of 30-day mortality in IPD patients.** We conducted an unbiased association study for the presence or absence of pneumococcal genes and mortality within the first 30 days of hospitalization (Figure 1A). Analysis was performed on 349 sequenced pneumococcal isolates, collected from a clinical IPD cohort, which comprised of strains from multiple lineages (10) (Figure 1B). GWAS was stratified for population structure, whereby the sequence cluster membership as determined by Bayesian Analysis of Population Structure (BAPS) was used as a covariate in a Cochran–Mantel–Haenszel test (11). The overall 30-day mortality within this IPD cohort was 11% (37/346, outcome unknown for 3 cases). We observed that out of the 1946 orthologous genes (OGs) of the pneumococcal accessory genome, *pblB* had a strong statistical correlation with 30-day mortality, with a Bonferroni corrected p-value of 0.00034, and was present in 48% of the 349 clinical isolates.

We identified *pblB* as the phage-derived gene potentially most relevant in the pathophysiology of IPD through its interaction with platelets although it co-occurred with other phage genes (Table S1). Sequence examination of a representative clinical isolate PBCN0103 revealed that two copies of *pblB* were located within the same phage element next to OG_175 (holin) and OG_675 (hypothetical protein), which both co-occurred with *pblB* and were also significantly associated with 30-day mortality (Figure S1). In addition, OG_58, located in a different phage operon than the aforementioned genes, is also significantly associated with 30-day mortality (Table S1). Strikingly, these 4 OGs were present simultaneously in 168 out of 349 isolates (Figure S2).

Among the IPD cases caused by pneumococci containing the *pblB* gene (*pblB*$^+$), 27 out of 165 died within 30 days (16.4%), compared to only 10 out of 181 (5.5%) caused by those not containing the *pblB* gene (*pblB*$^-$) (p=0.0011; OR 3.3). In a sub-analysis of cases who died without any limitations of medical treatment, 30-day mortality was 15/165 (9.1%) in *pblB*$^+$ and 6/171 (3.3%) in *pblB*$^-$ cases, which remained statistically significant (p=0.022; OR 2.8). For all cases, the presence of *pblB* was an independent determinant of 30-day mortality (OR 3.4, 95% CI: 1.5-7.6), next to Charlson comorbidity index score (OR 1.5, 95% CI: 1.2-1.7) and meningitis (OR 4.6, 95% CI: 1.6-13.7). For pneumonia cases separately, in addition to Pneumonia Severity Index (PSI) score (OR 1.4, 95% CI: 1.1-1.7) and Charlson comorbidity score (OR 1.02, 95% CI: 1.01-1.04), both designed to predict mortality, the presence of *pblB* was an independent risk factor for 30-day mortality (OR 3.4, 95% CI: 1.2-9.5).

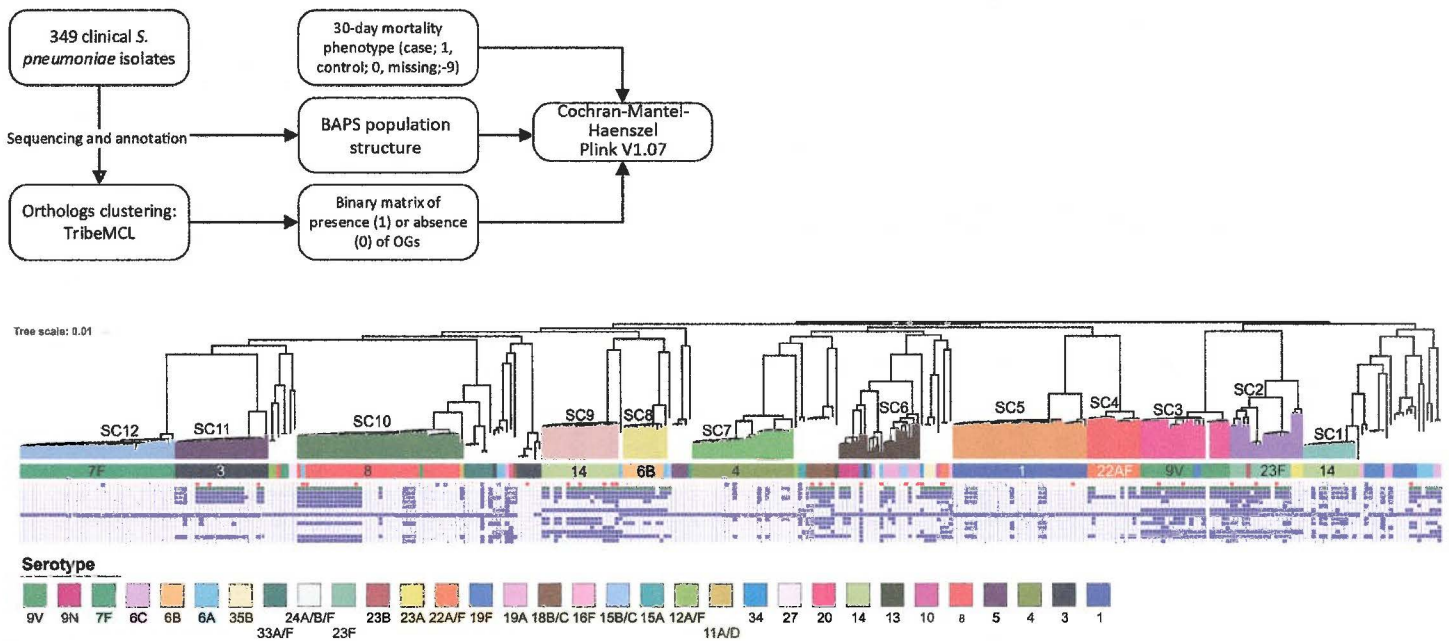

Figure 1A. Flowchart of the computational method used to identify the association between the presence of orthologous genes (OGs) and 30-day mortality. B. A phylogenic tree of the variable sites from the core genome of all blood clinical isolates used in study.

Phylogeny and sequence clustering were obtained from Cremers et al. (35). Pneumococcal clades are colored according to their sequence clusters. Filled squares: present, open squares: absent. In red: 30-day mortality. In dark green: pblB. In blue: other significantly associated genes (top to bottom: OG_675, OG_58, OG_1885, OG_2439, OG_1220, OG_558, OG_2259, OG_866, OG_2298, OG_1029, OG_175 [phage holin], OG_2232).

Fluoroquinolones induced the expression of *pblB*. It was unknown whether *pblB*-containing temperate pneumophages are specifically induced by this group of antibiotics *in vitro*. Therefore, different doses of ciprofloxacin (CPX) and levofloxacin (LVX); both belonging to the fluoroquinolone group of antibiotics), mitomycin C (MitC) and penicillin G (PenG; a beta-lactam antibiotic) were tested on three *pblB*-containing pneumococcal strains (PBCN0103, PBCN0226, PBCN0239) in THY medium to determine the sublethal dose of the four antibiotics (data not shown). To confirm that the selected doses were not bactericidal, the number colony forming units (CFU) were determined after exposure of MitC, PenG and the fluoroquinolones for two hours at 37 °C and 5% CO_2 (Figure S3). At the same time point, the difference in expression of *pblB*, relative to *gyrA*, was measured. The DNA cross-linking agent MitC was included as positive control. Both the fluoroquinolones CPX and LVX (data not shown) induced the expression of *pblB*, which appeared specific for this group of antibiotics, as the beta-lactam antibiotic PenG did not induce the expression. Furthermore, strong variation was found between the different pneumococcal strains (Figure 2).

Figure 2

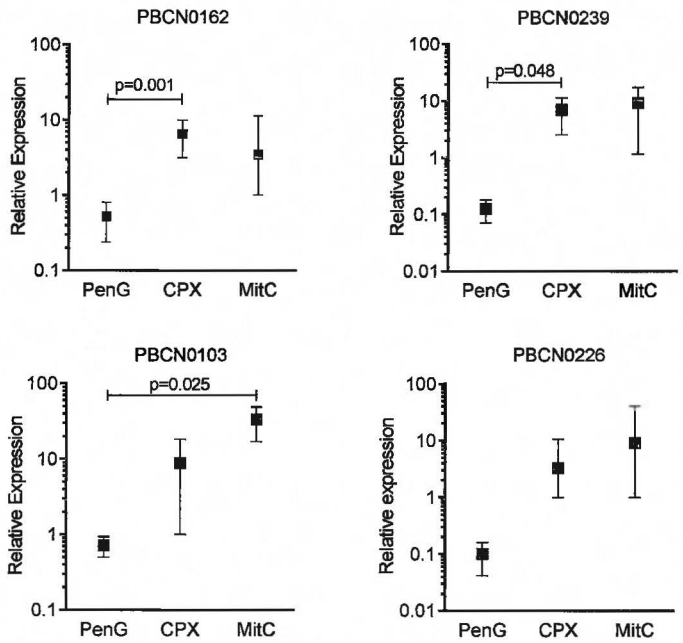

Figure 2. Sublethal doses of antibiotics induced pneumococcal expression of the pblB phage in culture medium.

Induction of pblB expression after two hours of incubation with sub-lethal doses of antibiotics were determined in 4 different pneumococcal clinical isolate strains (PBCN0162, PBCN0239, PBCN0103 and PBCN0226) by qRT-PCR measuring levels of mRNA relative to the control gyrA. Data presented are means with 95% confidence interval from three independent experiments.

Simulation of the clinical conditions in a whole blood *ex vivo* assay. Of the 312 patients with sequenced strains and known empirical treatment, 28% (n=88) received only beta-lactam, 4% (n=11) received only fluoroquinolones, and 44% received a combination of a beta-lactam and a fluoroquinolone. To simulate the aforementioned clinical conditions, we incubated live pneumococci strain PBCN0162 containing a mutationally-inactivated *pblB* gene (Δ*pblB*) or wild type (wt), with and without antibiotics (PenG, CPX, and a combination of PenG and CFX) in whole blood, determined the expression of *pblB* using qPCR (Figure 3A) and measured in the same samples the activation of platelets. We were able to measure *pblB* expression of the wt pneumococci in the whole blood samples without antibiotics (mean Cq value 30.6, 95% confidence interval 29.5-31.7) and its increase in the presence of antibiotics. We first analyzed whether the different antibiotics significantly affect the wt-/Δ*pblB*-bacteria-mediated platelet activation state in whole blood using a liner mixed model. We found that in all cases, stronger activation of platelets was observed with wt pneumococci as compared to Δ*pblB*, which clearly indicates that PblB induces enhanced platelet activation irrespective of the exposure to antibiotics (Figures 3B).

Whilst PenG did not strongly induce expression of *pblB* in THY-medium-grown pneumococci (Figure 2B), we observed PenG-dependent induction (~3-fold) of expression in whole blood (Figure 3A). This might be caused by an indirect effect as a consequence of the bactericidal effect of PenG leading to the production of reactive oxygen species (ROS) which has DNA damaging effects, inducing the expression of *pblB*. Despite the fact that expression of *pblB* was much stronger in whole blood containing CPX, platelet activation is not increased accordingly, indicating a close to maximum activation under these conditions.

Figure 3

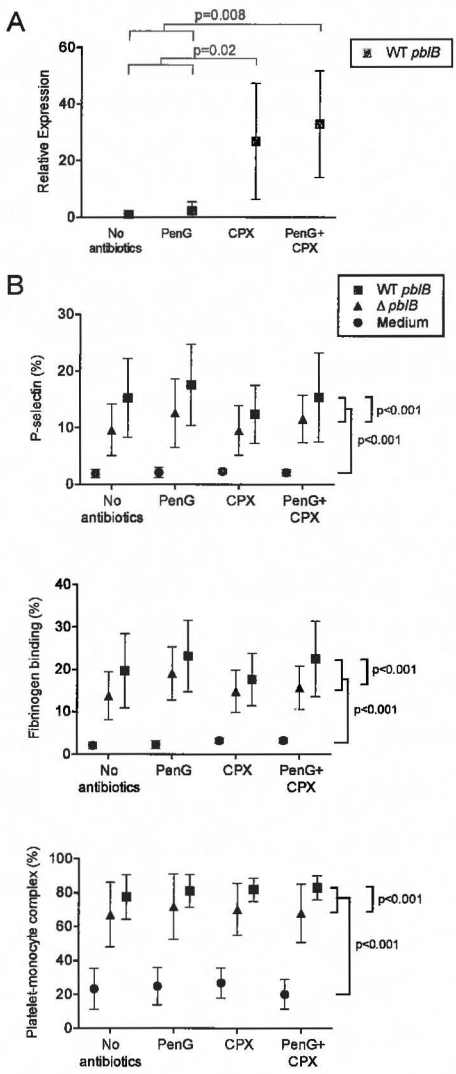

Figure 3. Live wild type pneumococci in an *ex vivo* whole blood assay showed increased expression of *pblB* upon exposure to fluoroquinolones and induced higher platelet activation as compared to the pblB knock-out mutant, irrespective of antibiotics exposure.

Live pneumococci (strain PBCN0162) containing a mutationally-inactivated *pblB* gene (Δ*pblB*) or wild type (WT *pblB*) were incubated in whole blood in the presence or absence of penicillin G (PenG), ciprofloxacin (CPX), or a combination of both. After two hours of incubation at 37 °C and 5 % CO_2 the expression of *pblB* was determined using qRT-PCR measuring levels of mRNA relative to the control samples. One extreme outlier value, as determined by the Grubbs' test, in the CPX-exposed sample was excluded from the figure (A). Platelet expression of P-selectin, platelet-fibrinogen binding and platelet-monocyte complex formation were measured in the same samples using flow cytometry and expressed in percentage of positivity (B). Data presented are means with 95% confidence interval from three independent experiments with blood derived from a total of 6 human volunteers.

Discussion

In the present study, GWAS was performed using the sequences of 349 *S. pneumoniae* invasive disease isolates to test for associations between the presence or absence of genes in the pneumococcal accessory genome and 30-day mortality. The presence of the phage-encoded *pblB* gene was positively associated with 30-day mortality in patients with IPD. This finding suggested the role of *pblB* in the pathogenesis and expected cause of death of IPD. The presence of the *pblB* phage gene as risk factor remained after adjustment for the local pneumococcal population structure using BAPS. We therefore speculate that similar studies in other areas with different pneumococcal populations would yield similar findings, although this requires confirmation by other studies. The *pblB* phage in our cohort was barely present in serotypes 1 and 7F, which are associated with a lower risk of death than other serotypes (12).

Past observations reported that 75% of pneumococcal clinical isolates carry bacteriophages (pneumophages) (13), which may be distributed among pneumococcal isolates with different capsular serotypes, indicating that these mobile genetic elements are widely spread among clinically relevant pneumococcal strains (14). The hypothesis that bacteria acquire virulence properties from phages is widely accepted (15), however, there has been a paucity of data supporting the role of bacteriophages in the pathogenesis of *S. pneumoniae*-caused diseases. Interestingly, *pblB* co-occurred with two other genes in the phage element, one encoding for a hypothetical protein and the other for Holin, both of which were also found to be significantly associated with 30-day mortality. The simultaneous co-occurrence of these genes in almost 50% of our clinical isolates further indicates a functional link between PblB expression and 30-day mortality. Holin is involved in the release and mounting of PblB on the bacterial surface of *S. mitis* (16), allowing the PblB interaction with and propagation of platelet activation. Furthermore, PblB expression was also found to contribute to virulence in an *in vivo* rabbit model of infective endocarditis (17)(16). These findings indicate that *pblB* has an important role in endovascular infection.

Most patients in this cohort were treated with a combination of penicillin and ciprofloxacin, which represented a common first line empiric antibiotic regime for severe community acquired pneumonia in the Netherlands (18). We therefore proceeded with *ex vivo* experiments in which live pneumococci were incubated in whole blood supplemented with penicillin or ciprofloxacin or a combination of the two, to simulate the clinical conditions. The wild type pneumococci clearly demonstrated enhanced platelet activation. Interestingly, there were differences in platelet activation between knock-out mutant and wild type pneumococci even in the absence of high *pblB* induction by the antibiotics. This may be explained by a constitutive expression of *pblB*, which despite its low level was sufficient to induce platelet activation, as had been

described in *S. mitis* (17).

S. pneumoniae has been shown to directly activate platelets mainly through TLR2 (19), with FcγRIIA and integrin αIIbβ3 being involved in the *amplification* of bacteria-induced *platelet* activation (20). This leads to platelet degranulation and subsequently the release of an array of chemokines and inflammatory mediators which may modulate not only their own function but also cells around them (21, 22). Our findings that whole blood exposure to WT pneumococci result in higher platelet activation compared to the *pblB* knock-out mutant may explain why bacteremic patients, infected with pneumococci containing the *pblB* gene, have a higher chance to die within 30 days. An approximate of 20% increase from baseline values in platelet P-selectin expression and PMC has been associated with adverse cardiovascular events and the acute phase of ischaemic stroke (23, 24), and the increase in platelet activation associated with *pblB* in our *ex vivo* assays exceeded this aforementioned value. By causing enhanced platelet activation, bacteria can become engulfed in a septic thrombus and protected from other cells of the immune system, allowing them to persist in the circulation (25). We speculate that the *pblB*-enhanced platelet activation may confer this survival advantage for *S. pneumoniae*. On the other hand, the resulting excess of platelet activation together with platelet clumping, platelet-leukocyte and platelet-endothelium aggregation and increased fibrin formation result in enhanced thrombo-inflammatory responses, microvascular obstruction, tissue hypoperfusion and finally multi-organ failure in sepsis (26, 27). The increase of PMC formation predicts mortality in older septic patients (28), and platelet consumption associated with platelet activation in sepsis patients leads to thrombocytopenia, which has been shown to increase the risk of mortality (29-31).

Our results have several potential clinical implications. We found that the presence of *pblB* was an independent determinant of 30-day mortality, which illustrates that bacterial GWAS potentially identifies intra-species variation related to clinical risks associated with human infection. Knowledge of the bacterial genotype might improve clinical management, by increasing alertness for a particular disease manifestation, in this case, diffuse intravascular coagulation in *pblB*-positive IPD patients. However, as disease manifestations are generally the product of multiple covariates, the contribution of bacterial genotype may vary across clinical settings. Secondly, our results demonstrated that fluoroquinolones induce higher *pblB* expression. However, the presence of fluoroquinolones was not required by the *pblB*-expressing wild type pneumococci to enhance platelet activation when compared with the knock-out mutant. Given that fluoroquinolones are frequently used in the management of community-acquired pneumonia for the coverage of atypical pathogens (32), sufficiently-powered studies are needed to investigate the clinical outcomes of the interplay between antibiotics regimen and *pblB* before drawing any conclusions. Thirdly, our study further highlights the importance of platelet-bacterial interaction and platelet activation, both in providing

a survival advantage for bacteria and in posing increased risk of mortality in patients. There is more and more data on the use of platelet function inhibitors in sepsis, however, these results at times contradict (9). Platelet inhibition by the $P2Y_{12}$ receptor antagonists reduces the release of pro-inflammatory mediators from the platelet α-granules (33). Taken together with our findings, the benefit of anti-platelet agents as adjunctive therapy in sepsis warrants further investigation.

The limitation of our study is the paucity of information on PblB protein expression on the pneumococcal surface. Previously, PblB of *S. mitis* was shown to function in adhesion by interacting with α 2-8-linked sialic acid residues on platelet membrane gangliosides (34). More recently, Hsieh and colleagues showed that *pblB* knock-out mutant pneumococci had decreased adherence to respiratory epithelial cells and platelets (8). Further work to demonstrate pneumococcal *pblB* expression at the protein level, as well as identifying its binding domain on platelets, is needed. To the best of our knowledge, this is the only patient-based study which reveals the role of *pblB* gene expression in the pathogenesis of IPD based on an extensive analysis of both bacterial genomics and clinical data, independently adding substantial evidence to only two previous studies on pneumococcal *pblB* *in vitro* and in mice (8, 35).

In conclusion, we have integrated genome sequencing and GWAS with functional characterization to investigate the clinical role of genomic *pblB* presence in the mortality of patients with IPD. Bacterial GWAS may be an important tool to study the potential predictive value of certain virulence genes. As genomic sequencing is increasingly being utilized, we believe that this integrated approach will assist greatly in elucidating the mechanisms of bacterial pathogenesis, leading to the development of novel diagnostics and new therapeutic approaches.

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Supplemental Material

Method S1.

Adjustment for covariates of mortality.

Certain patients had predetermined limitations of medical treatments, for example, opted not to be transferred to the intensive care unit. Therefore, the relation between OGs and 30-day mortality was also established separately for those who died after fully-applied treatment. For potential covariates of the associations between OGs and 30-day mortality, differences between patients with or without the OGs were assessed to decide which variables to include in the initial multivariable logistic regression model with 30-day mortality as dependent variable. The variables included were gender, age, year of inclusion, comorbidities (i.e. cancer, COPD, diabetes mellitus, liver-, renal-, cardiovascular- and cerebrovascular disease, Charlson comorbidity index score), clinical diagnosis, blood C-reactive protein level, presence of Systemic Inflammatory Response Syndrome (SIRS) and pleural effusion, Pneumonia Severity Index (PSI) score, admission to ICU, mechanical ventilation, and class of antibiotics administered. By manual stepwise backward elimination, the initial model was reduced to the final model, which only included covariates that contributed to the model with a p-value <0.10. These analyses were performed using IBM SPSS statistics version 23.

Table S1.

Associations between patients mortality within the first 30 days of hospitalization (30-day mortality), and the presence and/or absence of genes in the pneumococcal isolates (represented as orthologous groups;OG). The *p-values* are Bonferroni corrected for multiple testing and stratified for population substructure using BAPS clusters [See material and methods].

OG_ID	Annotation (Truncated)	Prevalence	Corrected p-value
OG_675	Hypothetical protein	163	0.000228
OG_17	Phage hyaluronidase PblB	166	0.000344
OG_58	Phage protein	177	0.001017
OG_1885	Phage protein (prophage LambdaSa2)	85	0.001923
OG_2439	Hypothetical protein	41	0.00201
OG_1220	Phage protein	93	0.002032
OG_558	ROK family protein	318	0.002231
OG_2259	Hypothetical protein	64	0.002756
OG_866	Phage protein	106	0.003589
OG_2298	Phage protein	27	0.003685
OG_1029	Hypothetical protein	78	0.003737
OG_175	Phage holin	171	0.004332
OG_2232	DNA-binding protein	41	0.045225

Table S2. List of primers

Target region	Primer
CvdG_pblB_PBCN162_L1	CGTTATCCAAATCGCAGGAC
CvdG_pblB_PBCN162_L2	CCACTAGTTCTAGAGCGGCGGCTCTGTCAAATTGTCGTC
CvdG_pblB_PBCN162_R1	AATTGGCGACGGCCATTTAC
CvdG_pblB_PBCN162_R2	GCGTCAATTCGAGGGGTATCGACTATACCGCTTTAGTTCC
PBpR412_L	GCCGCTCTAGAACTAGTGG
PBpR412_R	GATACCCCTCGAATTGACGC

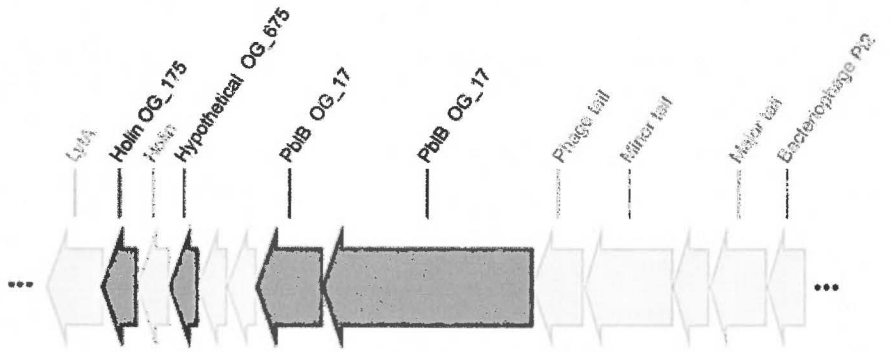

Figure S1. Part of the operon of a phage element.
Genes are directed in reverse. Labels indicate gene products and unlabeled arrows indicate hypothetical gene products. A total of 349 clinical isolates from patients with IPD were sequenced, annotated and the genes were clustered into orthologous groups (OG). Sequence examination of a representative clinical isolate PBCN0103 revealed that two copies of *pblB* are located within a phage element in next to OG_175 (holin) and OG_675 (hypothetical protein), both of which were also associated with 30-day mortality.

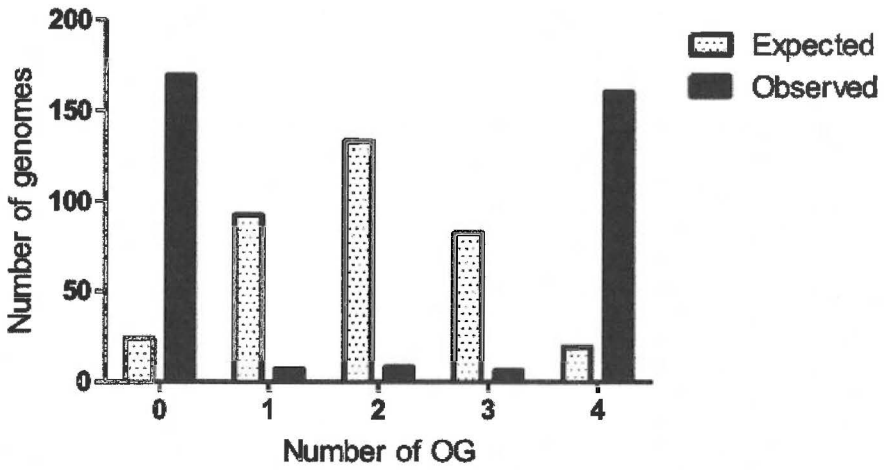

Figure S2. Co-occurrence of *pblB* with other OGs associated with 30-day mortality.
Thirteen OGs were statistically associated with 30-day mortality, of which four, namely OG_17 (*pblB*), OG_175 (holin), OG_675 (hypothetical protein) and OG_58 (phage protein), were present simultaneously in 168 out of the 349 pneumococcal genomes. We tested whether these OGs co-occur within the same clinical isolates rather than being randomly distributed. The number of isolates that contain zero, one, two, three or all four of these OG(s) simultaneously was counted ("Observed"). The expected co-occurrence of the four OGs over the 349 genomes was mathematically calculated by multiplying the probability that a randomly picked genome contains 0, 1, 2, 3 or all 4 OGs with the total number of genomes (349) ("Expected").

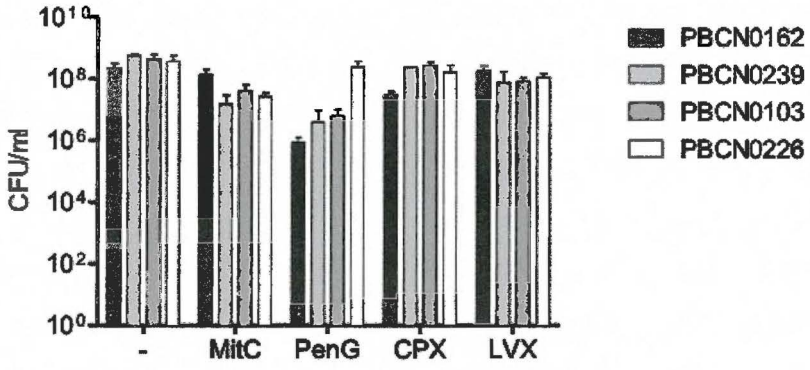

Figure S3.

Average CFU values were determined after incubation of the three clinical pneumococcal strains (PBCN0162, PBCN0239, PBCN0103, PBCN0226) for two hours at 37°C and 5% CO_2 in THY medium with sub-lethal doses of antibiotics; mitomycin C (MitC), penicillin G (PenG), ciprofloxacin (CPX) and levofloxacin (LVX). The condition without antibiotics (-) was included as negative control.

Chapter 8

Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis

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Abstract

Platelets may play a role in the high risk for vascular complications in Gram-positive sepsis. We compared the platelet reactivity of 15 patients with Gram-positive sepsis, 17 with Gram-negative sepsis and 20 healthy controls using a whole blood flow cytometry-based assay. Patients with Gram-positive sepsis had the highest median fluorescence intensity (MFI) of the platelet membrane expression of P-selectin upon stimulation with high dose adenosine-diphosphate (ADP; P=0.002 vs. Gram-negative and P=0.005 vs. control groups) and cross-linked collagen-related peptide (CRP-XL; P=0.02 vs. Gram-negative and P=0.0001 vs. control groups). The Gram-positive group also demonstrated significantly higher ADP-induced fibrinogen binding (P=0.001), as well as platelet-monocyte complex formation (P=0.02), compared to the Gram-negative group and had the highest plasma levels of platelet factor 4, β-thromboglobulin and soluble P-selectin. In contrast, thrombin-antithrombin complex and C-reactive protein levels were comparable in both patient groups. In conclusion, common Gram-positive pathogens induce platelet hyperreactivity which may contribute to a higher risk for vascular complications.

Introduction

The concept that platelets play an important role in immunity and host defence, besides their role in haemostasis, has become widely accepted. Many different bacteria, viruses and other micro-organisms have been shown to interact with platelets, changing the platelet phenotype and the interaction of platelets with leukocytes [1, 2]. Whereas platelet activation is important in innate immune responses, excessive platelet activation may contribute to organ dysfunction and increase the risk for cardiovascular events (CVE) and infective endocarditis [3, 4]. Acute bacterial infections are associated with an increased risk for CVE, but the magnitude of this risk, as well as the risk for infective endocarditis, depends on the type of infections [5-7].

Bacteria are generally divided in Gram-positive and Gram-negative bacteria. In contrast to Gram-negative bacteria, Gram-positive bacteria usually have a thick multi-layered peptidoglycan cell wall rich in teichoic acid with virtually absent lipopolysaccharide (LPS) [8]. Clinical studies have shown a high incidence of cardiovascular complications in patients with Gram-positive bacterial infection [9, 10]. This risk appears lower for urinary tract infections, which are usually caused by *Escherichia coli* or other Gram-negative bacteria [5, 11]. In addition, infective endocarditis is predominantly caused by Gram-positive bacteria [12].

Laboratory studies have shown that Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus* spp., bind to and activate platelets, whereas this has not been demonstrated for most Gram-negative bacteria [13]. Interestingly, our group recently demonstrated that bacteremia due to *Streptococcus pneumoniae* in pigs lead to platelet hyperreactivity [14]. On the other hand, data on the platelet-activating properties of *E. coli* LPS, which exerts potent inflammatory effects, have been conflicting and challenging to translate to the human *in vivo* setting [15-18].

Studies on the platelet-activating properties of bacteria were most frequently done either *ex vivo* or in animal models. To the best of our knowledge, no study has provided human *in vivo* data comparing platelet reactivity in patients with Gram-positive or Gram-negative sepsis. We therefore studied platelet reactivity, platelet-monocyte complex (PMC) formation and activation of the plasmatic coagulation in a cohort of patients with either Gram-positive or Gram-negative sepsis.

Methods

Study population

We enrolled adult patients admitted with a Gram-positive or Gram-negative sepsis in the Radboud university medical center, Nijmegen, the Netherlands. A list of patients with positive blood cultures was generated daily by the Department of Medical Microbiology and patients were reviewed for in- and exclusion criteria. Blood was drawn for platelet reactivity and haemostasis tests within 72 hours after the first blood culture collection and initiation of antibiotic treatment. Exclusion criteria were admission to the intensive care unit (ICU), renal replacement therapy, use of $P2Y_{12}$ receptor antagonists, active malignancy, the presence of any chronic viral infection, including HIV or hepatitis B/C and a positive blood culture with a possible contaminant (e.g. coagulase-negative staphylococci). ICU patients were deliberately excluded to avoid confounding of organ failure on the assessment of the effects of bacteremia on platelet reactivity. Use of a low dose of aspirin or vitamin K antagonists was not a reason for exclusion, as these drugs have no influence on the platelet reactivity assay used [19, 20]. A group of healthy volunteers were enrolled as controls. Patients and healthy controls were included after written informed consent was obtained. The study is approved by the ethical committee of the Radboud university medical center.

Laboratory assays

Platelet reactivity

Venous blood was collected in citrated Vacutainer tubes (3.2% sodium citrate; Becton Dickinson, USA). Platelet reactivity was determined within 1 hour after blood drawing by a flow cytometry assay that was described earlier [21, 22]. In short, the expression of the α-granule protein P-selectin and binding of fibrinogen to the activated integrin αIIbβ3 are measured as markers of platelet degranulation and aggregation, respectively, in unstimulated samples and after *ex vivo* platelet stimulation by adenosine diphosphate (ADP, low dose of 7.8 μM and high dose of 31.2 μM, Sigma-Aldrich, USA) or cross-linked collagen-related peptide (CRP-XL, low dose of 39 ng/L and high dose of 625 ng/L, kind gift from Prof. dr. R. Farndale, Cambridge, UK). Whole blood was added to a mixture of HEPES-buffered saline and saturating concentrations of PE-labeled anti-CD62P (P-selectin; Bio-Legend, San Diego, USA), FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK) and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). After 20 minutes incubation at room temperature, 0.2% paraformaldehyde was added and samples were analyzed using an FC500 flow

cytometer (Beckman Coulter, France). Platelets were gated based on their forward- and sideward-scatter properties and positivity for CD61, which was defined as a median fluorescence intensity (MFI) exceeding that of its matched isotype control. Next, the MFI of CD62P and fibrinogen relative to their matched isotype controls on CD61-positive events was determined.

Platelet-monocyte complexes

The formation of PMC, which is considered a sensitive marker for platelet activation [23], was determined by incubating citrated whole blood with PC7-labelled anti-CD61 and PE-labelled anti-CD14 [a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein; Bio-Legend] as a monocyte identification marker. Optilyse B, which contains both lysing buffer and fixative (Beckman Coulter, USA) was added after 30 min followed by distilled water. The PMC formation was quantified based on the MFI of CD61 on CD14-positive cells.

Soluble platelet proteins and thrombin-antithrombin complexes

Platelet-poor plasma was harvested from citrate-anticoagulated whole blood by centrifugation (1500 g without brake, 15 min, 20°C). Plasma concentrations of platelet factor 4 (PF4), b-thromboglobulin (b-TG), soluble P-selectin and thrombin-antithrombin (TAT) complexes were subsequently measured using ELISA as previously described [24]. Human PF4 (MAB7951, AF795), β-thromboglobulin (MAB393, BAF393) and soluble P-selectin (DYE137) antibodies were purchased form R&D systems, Abbington, UK. Sheep anti-human thrombin (SAHT-AP, SAHT-HRP) antibodies were purchased from Kordia/Affinity Biologicals, USA.

Full blood count and C-reactive protein

A full blood count was determined using a standard hematology analyser (Sysmex XE 5000) calibrated for standard patient care. C-reactive protein (CRP) was determined using immunologic agglutination detection and the urinary concentration of creatinine was determined with enzymatic colorimetric detection using Abbott Aeroset analyzer (Abbott Laboratories).

Statistical analysis

Differences in patient characteristics across groups were compared using analysis of variance (ANOVA) with post-tests and chi-square test for proportions. Data on platelet reactivity are expressed as medians with interquartile range (IQR), while data on plasma soluble markers and PMC formation are expressed as medians with IQR, minimum and

maximum values. The Mann-Witney U test was used to analyse statistical differences in the time intervals between the haemostatic tests and antibiotic treatment initiation or blood culture, in the CRP levels among the patient groups and in the platelet reactivity of patients with or without aspirin. Platelet reactivity and plasma markers levels between multiple groups were compared using the non-parametric Kruskal–Wallis test with Dunn's post-test. Analyses were performed with GraphPad Prism (GraphPad Software, USA). *P* values less than 0.05 were considered statistically significant.

Results

Thirty-two patients with sepsis were enrolled, of whom 15 had a Gram-positive and 17 a Gram-negative sepsis, with 20 healthy individuals as controls (Table 1). Patients in the Gram-positive group presented clinically with sepsis (n=8), erysipelas (n=3), pneumonia (n=2), and infected diabetic foot ulcer (n=1). The most common clinical diagnosis in the Gram-negative group was urosepsis (n=11), followed by cholecystitis and *intravascular catheter-related* infection (each n=2), as well as endometritis and infected diabetic foot ulcer (each n=1). Patient characteristics in both groups were similar, including time intervals between the haemostatic tests and antibiotic treatment initiation or blood culture (Fig 1A). The levels of CRP, measured on the day (± 24 hrs) when haemostasis assays were also performed, were comparable across both patient groups (Fig 1B). The mean age of the controls was lower than the patients in both sepsis groups.

Table 1. Characteristics of study population

	Gram-Positive	Gram-Negative	Healthy controls	*P* value
Number	15	17	20	
Male, n (%)	12 (80)	11 (65)	12 (60)	0.99
Age, years	67 (8)	70 (13)	31 (8)	<0.001*
Haemoglobin (g/dL)	7.6 (1)	7.2 (0.9)	ND	0.24
Leukocytes (x109/L)	12.2 (4.4)	10.4 (4.4)	ND	0.26
Platelets (x109/L)	178 (45)	202 (110)	ND	0.44
Creatinine (mg/dL)	98 (27)	118 (57)	ND	0.23
C-reactive protein (mg/L)	163 (97)	136 (85)	ND	0.41
Aspirin, n (%)	4 (27)	6 (35)	0 (0)	0.30
Vitamin K antagonist, n (%)	3 (20)	4 (24)	0 (0)	0.41
Causative pathogens, n (%)				
Gram-positive				
Staphylococcus aureus	10 (67)			
Streptococcus	2 (13)			
pneumoniae				
Group G Streptococcus	2 (13)			
Group C Streptococcus	1 (7)			
Gram-negative				
Escherichia coli		12 (71)		
Citrobacter spp.		1 (6)		
Haemophilus influenzae		1 (6)		
Klebsiella oxytoca		1 (6)		
Serratia spp.		1 (6)		
Bacteroides fragilis		1 (6)		

Data depicted are means with SD unless otherwise indicated. ND, not determined. Statistical differences are analysed by using ANOVA with post-tests or chi-square test. **P* values were statistically significant for differences between healthy controls vs. Gram-positive and Gram-negative groups.

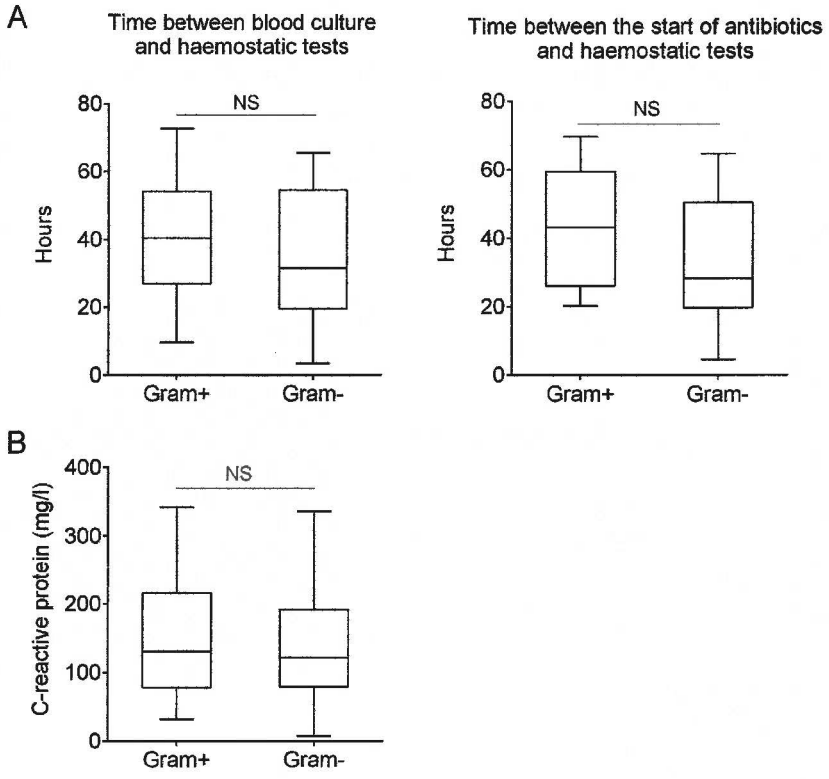

Figure 1. Timing of haemostatic tests and plasma concentration of C-reactive protein (CRP). Box plots of the (A) time interval between platelet reactivity and haemostatic test and positive blood culture (left panel) or start of antibiotics (right panel). (B) CRP levels at the day (+/- 24 hrs) of the haemostasis assays. Presented data are medians with IQR, minimum and maximum values. NS, not statistically significant.

Participants in the Gram-positive group had a significantly higher ADP- and CRP-XL-induced P-selectin expression than participants in the Gram-negative group and controls (Fig 2A). Upon stimulation with high dose ADP (31.2 uM), the Gram-positive group demonstrated the highest MFI of P-selectin (median 49.2, IQR 34.6 – 55.2) compared to the Gram-negative (28.1, 25.3 – 38.3; *P*=0.002) and control groups (29.7, 26.2 - 35.7; *P*=0.005). When induced with high dose CRP-XL (625 ng/ml), patients in the Gram-positive group had a significantly higher P-selectin (89.8, 72.4 – 95.4) than both the Gram-negative (69, 67.7 – 80.3; *P*=0.02) and control groups (67.6, 64.3 – 72.4; *P*=0.0001). ADP-induced platelet-fibrinogen binding was also significantly higher in this group (18.3, 15.6 – 24.2 vs. 12.6, 10.8 – 13.9; *P*=0.001 vs. Gram-negative group). There were no differences in these parameters between the Gram-negative group and the controls.

The Gram-positive group had significantly higher PMC formation compared to Gram-negative group. Median (IQR) MFI values of the platelet marker CD61 on CD14-positive cells were 15.6 (13.7 - 17.1) in the Gram-positive group compared to 8.5 (8.1 - 9.5; *P*=0.02)) in the Gram-negative group and 3.1 (2.0 - 6.5; *P*<0.0001) in controls (Fig 2B). The difference between the Gram-negative group and controls was also significant (*P*=0.004). There was a weak positive correlation between the platelet expression of P-selectin and PMC formation in the patients (Spearman, R^2=0.22; *P*=0.008)

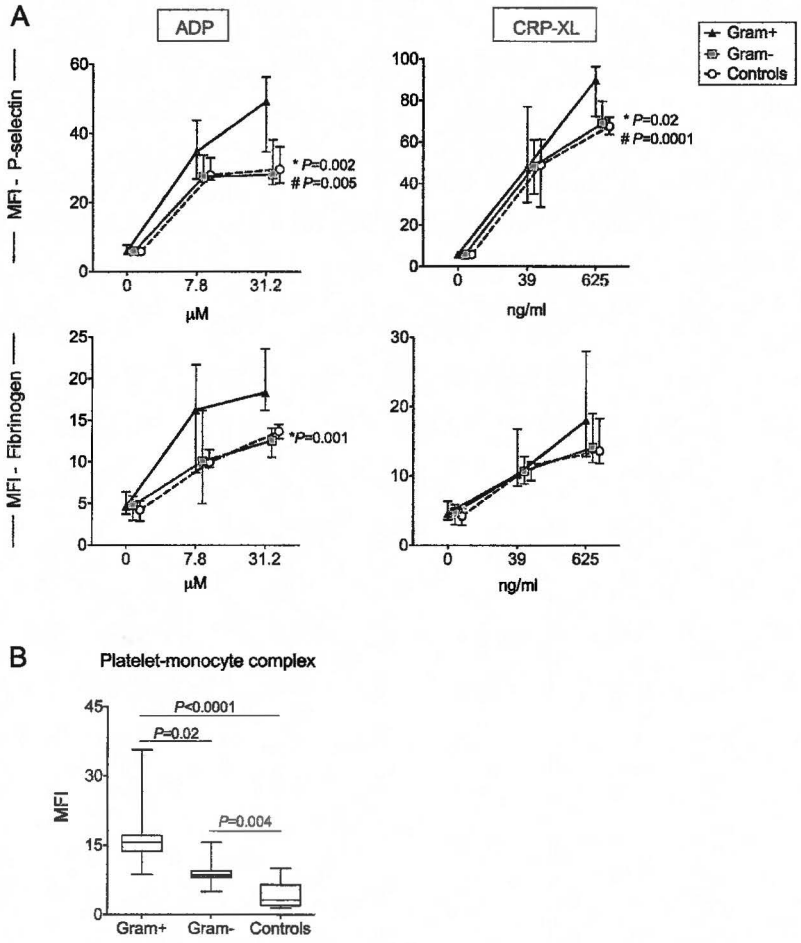

Figure 2. Platelet reactivity and platelet-monocyte complex in patients with Gram-positive and Gram-negative sepsis.

(A) Platelet membrane expression of P-selectin and platelet-fibrinogen binding is depicted as median fluorescence intensity (MFI) in arbitrary units, at baseline and after stimulation with two concentrations of the platelet agonists adenosine diphosphate (ADP) and collagen-related peptide (CRP-XL) in healthy controls (n=20), patients with Gram-positive (Gram+, n=15) and Gram-negative (Gram-, n=17) sepsis. (B) Platelet-monocyte complex (PMC) formation is depicted as the MFI of the platelet marker CD61 on CD14-positive cells. Data depicted are medians with IQR (platelet reactivity) or median with IQR, minimum and maximum values (PMC). * Gram-positive vs. Gram-negative, #Gram-postive vs. healthy controls.

Activated platelets release their α-granule contents, including P-selectin, PF4 and β -TG in the plasma. Plasma concentrations of these proteins were significantly higher in the Gram-positive group compared with the Gram-negative group and the healthy controls. Median (IQR) concentrations of PF4 in these respective groups were 32.1 ng/ml (18.6 - 46.2 ng/ml), 16.5 ng/ml (2.1 - 25.3 ng/ml; *P*=0.01 vs. Gram-positive) and 7.8 ng/ml (5.0 - 9.8 ng/ml; *P*<0.001 vs. Gram-positive). β-TG concentrations were 200.7 ng/ml (150.6 - 250 ng/ml), 126.5 ng/ml (77.1-174.9; *P*=0.038 vs. Gram-positive) ng/ml and 81.7 ng/ml (66.8 - 96.8 ng/ml; *P*=0.0008 vs. Gram-positive). P-selectin concentrations were 136.4 ng/ml (92.9 - 168.4 ng/ml), 111.2 ng/ml (96.7-137.1 ng/ml; *P*=0.61 vs. Gram-positive) and 84.4 ng/ml (72 - 99 ng/ml; *P*=0.015 vs. Gram-positive). The latter protein was the only soluble platelet parameter that was significantly higher in the Gram-negative group compared to controls (*P*=0.045). In contrast, there were no significant differences across the three groups in the plasmatic coagulation marker TAT complexes (Fig 3). In all patients, soluble P-selectin were weakly correlated with PMC formation (Spearman, R^2=0.23; *P*=0.01). Data of these plasma soluble markers were missing in 2 patients diagnosed with *S. aureus* sepsis in the Gram-positive group.

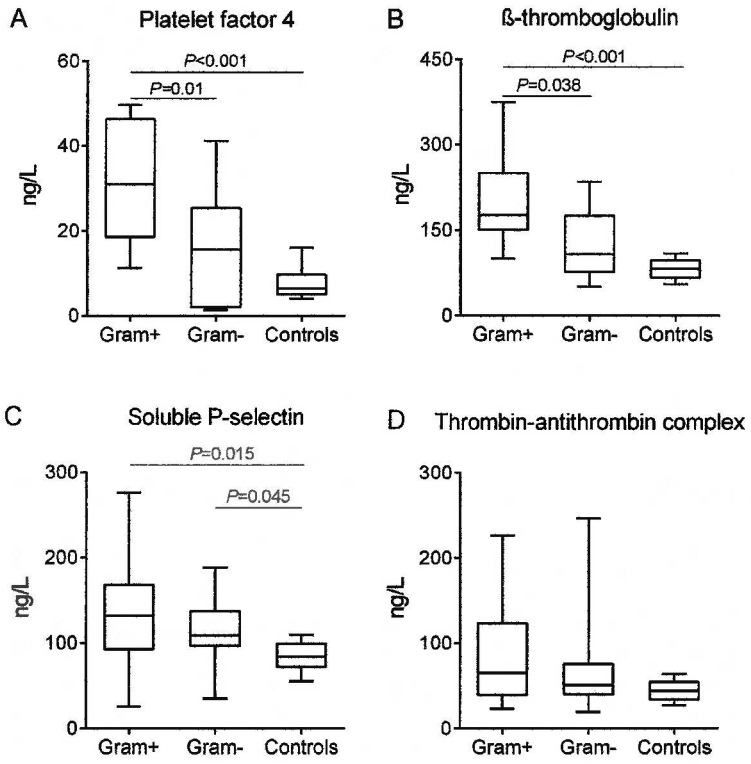

Figure 3. Plasma concentrations of platelet and coagulation activation markers.
Data depicted are medians with IQR, minimum and maximum values.

We performed subanalyses within the patient groups and found that those with a *S. aureus* or *Streptococcus spp* sepsis in the Gram-positive group had comparable platelet P-selectin expression upon induction with high dose ADP (MFI 47.4, 40.7 - 53.8 vs. 44.5, 26.9 - 58.2; *P*=0.67) and platelet-fibrinogen binding (MFI 19.0 , 14.7 – 24.0 vs. 17.4, 13.6 - 22.3; *P*=0.61), as well as PMC formation (18.3, 15.3 - 21.9 vs. 14.4, 12.1 - 16.5; *P*=0.26). Similarly, there were no differences in these parameters between patients with *E. coli* and other Gram-negative bacteria (data not shown). Aspirin treatment did not influence ADP- and CRP-induced platelet reactivity, PMC formation or the plasma soluble markers (data for ADP-induced platelet reactivity and PMC formation shown in Fig 4). There was no significant correlation between ADP-induced P-selectin expression and the time interval between haemostatic tests and the start of antibiotics administration (Spearman, R^2=0.06, *P*=0.07).

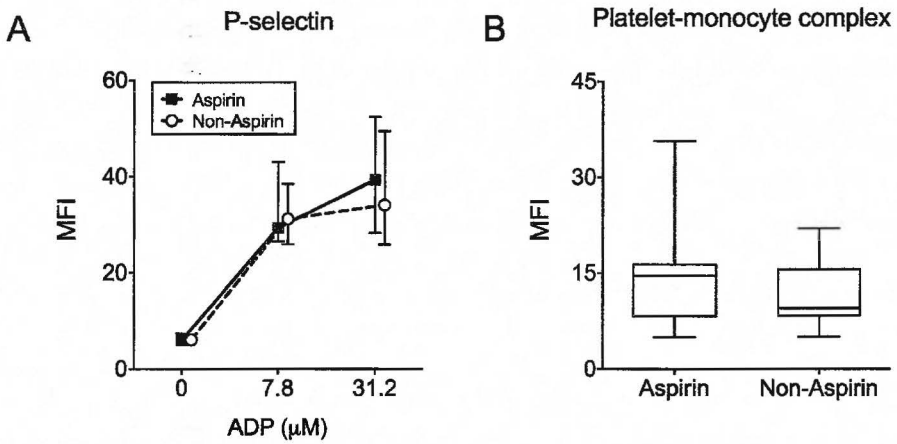

Figure 4. Platelet reactivity in aspirin and non-aspirin using patients.
Data depicted are medians with IQR, minimum and maximum values.

Discussion

Our data reveal that a sepsis with common Gram-positive pathogens is associated with more pronounced platelet activation, platelet hyperreactivity and PMC formation compared with sepsis due to common Gram-negative pathogens. These differences were observed despite comparable CRP plasma concentrations, suggesting that inflammation by itself is not a major driving force in sepsis-induced platelet activation. In contrast, there were no clear differences in the plasmatic coagulation parameter TAT complexes

between the Gram-positive and Gram-negative groups.
The majority of patients in the Gram-positive group had a *S. aureus* sepsis, whereas *E. coli* was the most common bacteria in the Gram-negative group. *S. aureus* and *Streptococcus spp.* are able to directly bind and activate platelets and different mechanisms through which these pathogens activate platelets have been reviewed recently by Hamzeh-Cognasse et. al. [13]. *S. aureus* can release toxins or use surface protein such as protein A and clumping factor A, which can subsequently bind platelet receptors directly or indirectly [25, 26]. Furthermore, FcγRIIa on platelets is essential for the amplification of their reactivity to these bacteria. The engagement of FcγRIIA by bacteria-bound plasma IgG, together with the integrin αIIbβ3 activation, results in integrin/FcγRIIA ITAM signalling which further triggers ADP, thromboxane A_2 (TxA_2) and PF4 release and leading to a strong positive feedback cascade of platelet activation [27]. Other *S. aureus* proteins, including lipoteichoic acid [28], inhibit platelet activation, although our present findings indicate that the platelet-activating potential of *S. aureus* are dominant *in vivo*. It was beyond the scope of our current study to characterize the pathways of platelet activation by the isolated pathogens. *E. coli* was also recently demonstrated to activate platelets in a FcγRIIa-dependent manner [29, 30], although *ex vivo* data of platelet activation by *E. coli* lipopolysaccharide are conflicting [15-17, 31]. Administration of lipopolysaccharide to healthy volunteers also does not appear to strongly activate platelets [32, 33], a finding supported by our data of limited platelet activation in patients with *E. coli* sepsis.

Vascular complications are common in patients with staphylococcal or streptococcal septicaemia [10, 34]. We speculate that the strong platelet-activating potential of these micro-organisms contributes to these complications. Platelet function inhibitors might reduce the risk for these complications [35], but this may not be without hazard, as activated platelets are able to limit the growth of and engulf *S. aureus* [36, 37]. In a mouse model, platelet inhibition also reduced killing of *S. aureus* [38, 39].
Limitations of our study include the limited number of patients in both groups and that the haemostatic assays were only performed once after the blood cultures became positive. Gram-negative bacteria can be rapidly killed by antibiotics and, even though no correlation existed between platelet reactivity and the time intervals between haemostatic tests and antibiotic treatment initiation, we cannot exclude that increased platelet activation was present in the Gram-negative group at presentation and that this normalized promptly after administration of antibiotics (i.e. before the blood culture became positive). Furthermore, many Gram-positive and Gram-negative bacteria exist and our findings cannot be extrapolated to other pathogens that were not included in this study, as their platelet-activating properties may differ. Finally, some patients used aspirin, which inhibits platelet activation via the COX-1 pathway [40]. This was, in

our study, not associated with decreased platelet reactivity via the ADP and collagen pathway or PMC formation.

In conclusion, Gram-positive sepsis is associated with marked platelet activation, in contrast to Gram-negative sepsis, and this may contribute to the vascular complications seen in these infections. Given the role of platelets in immunity and host defence, studies are currently undertaken investigating the possible role of platelet inhibition in sepsis patients [41]. It is important that causative pathogens are taken into account, as clear differences exist in the platelet-activating potential of bacteria, which influences the risk of vascular complications.

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Chapter 9

Reduced platelet hyperreactivity and platelet-monocyte aggregation in HIV-infected individuals receiving a raltegravir-based regimen

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Abstract

Objective: Platelets are key cells in atherosclerosis and acute cardiovascular events. Platelet hyperreactivity and increased platelet-monocyte aggregation (PMA) are found in HIV-infected patients and may contribute to the excess cardiovascular risk. The integrase inhibitor raltegravir (RAL) has been associated with better residual viral suppression and reduction in inflammatory and coagulation biomarkers. The aim of our study was to investigate whether RAL-treated patients have reduced platelet reactivity and PMA.

Design and methods: We performed a cross-sectional study involving 80 virologically suppressed adult HIV1-infected patients on a RAL-based (n=25), non-nucleoside reverse transcriptase inhibitor (NNRTI)-based (n=30) or a protease (PI)-based (n=25) regimen and 30 healthy controls. Platelet reactivity was determined by measuring platelet P-selectin expression and the binding of fibrinogen to platelets to stimulation with two concentrations of adenosine disphosphate (ADP). PMA was determined by measuring the expression of the platelet marker CD42b on CD14 positive cells.

Results: HIV-infected individuals had higher platelet reactivity and PMA than controls. RAL-treated individuals showed significantly lower P-selectin expression to stimulation with low (P=0.026 vs. NNRTI and P=0.005 vs. PI-group) and high dose ADP (P=0.009 vs. NNRTI and P=0.003 vs. PI-group). A similar trend for was found for fibrinogen binding although only the difference in P-selectin expression between RAL- and PI-treated patients reached statistical significance (P=0.038). PMA was also lower in the RAL group compared with the NNRTI (P=0.037) and PI (P=0.034) groups.

Conclusion: Use of a RAL-based regimen was associated with a reduction in persistent HIV-induced platelet hyperreactivity and PMA compared with NNRTI- and PI-based regimen.

Keywords: HIV, platelet, cardiovascular, raltegravir, platelet-monocyte aggregation

Introduction

Cardiovascular disease (CVD) has emerged as a leading cause of morbidity and mortality in HIV-infected individuals (1, 2). The precise mechanisms that contribute to this increased risk for CVD remain to be elucidated, but an important role is attributed to persistent immune activation and inflammation (3, 4).
Atherosclerosis is an inflammatory disorder, and platelet plays a pivotal role in its onset and progression (5). Inflammation activates platelets, which results in the release of a variety of inflammatory mediators that among others promote platelet adherence to the endothelium, leukocyte recruitment and formation of atherosclerotic lesions (6, 7). Platelet reactivity is helpful in predicting the risk of future cardiovascular events (8, 9), and activated platelets form aggregates with circulating monocytes, rendering them more proinflammatory and proatherogenic (10-14).
Increased platelet monocyte aggregation (PMA) and platelet reactivity to platelet agonists are found in HIV-infected patients (15-19). However, the impact of different regimens of antiretroviral therapy (ART) on platelet function and PMA has not been rigorously studied. Intensification of ART with the integrase inhibitor raltegravir (RAL) was recently shown to suppress residual viral replication and reduce plasma levels of the coagulation marker d-dimer (20). In addition, switching from a protease inhibitor (PI) or enfuvirtide to RAL decreased inflammatory and coagulation biomarkers (21). This suggests that a raltregravir-based regimen may also be associated with reduced platelet hyperresponsiveness and PMA. We aimed to investigate whether virologically suppressed HIV-infected patients using a RAL-based regimen have reduced platelet reactivity and PMA compared to those using a non-nucleoside reverse transcriptase reverse inhibitor (NNRTI)-based or PI-based regimen.

Methods

Study population and design
We performed a cross-sectional single cohort study enrolling adult HIV-infected patients during routine outpatient visits between November 2013 and April 2014 in the Radboud university medical center. All patients had a suppressed viral load (≤ 40 copies/mL) and were on a stable ART regimen for more than four months. The regimen consisted of a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) with one of the following options: RAL; an NNRTI (efavirenz, rilpivirin or nevirapine) or a ritonavir-boosted PI (darunavir, atazanavir, or lopinavir). Exclusion criteria were the use of aspirin or other platelet function inhibitors, as well as the presence of any other concurrent infection including active hepatitis B, C or syphilis infection. Data on platelet function and PMA in this cohort were compared with healthy adult volunteers of comparable age

and gender. The ethical committee of the Radboud university medical center approved this study and all participants provided written informed consent to participate.

Platelet reactivity and platelet-monocyte aggregation assay

Venous blood was collected in Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson). Baseline platelet activation status and platelet reactivity were measured using a novel flow cytometry-based assays which details were recently described (22, 23). In this assay, platelet membrane expression of the alpha-granule protein P-selectin (CD62P) and binding of fibrinogen to the activated $\alpha_{IIb}\beta_3$ receptor (the GPIIbIIIa complex) are measured as markers of platelet activation at baseline and after low (7.8 uM) and high dose (31.2 uM) of the platelet agonist adenosine diphosphate (ADP) with the following two combinations of PE-labeled anti-CD62P together with FITC-labeled anti-CD42b (anti-glycoprotein (GP)-1bα; platelet-identification marker; both antibodies from Bio-Legend, San Diego, USA) or FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK) with PE-labeled anti-CD42b (Bio-Legend, San Diego, USA). After incubation for 20 minutes at room temperature, 0.2% paraformaldehyde was added. Samples were analyzed with a Becton Dickinson flow cytometer. Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD42b, which was defined as a mean fluorescence intensity (MFI) exceeding that of the matched isotype control. Next, the MFI and percentage positivity of CD62P and fibrinogen on CD42b positive events were determined.

PMA was determined by incubating citrated whole blood with FITC-labeled anti-CD42b with PE-labeled anti-CD14 (a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein; Bio-Legend, San Diego, USA) as a monocyte identification marker. Optilyse B (Beckman Coulter, Fullerton, CA) and distilled water were added after 30 minutes. PMA was determined using the proportion of CD14+ cells that were also positive for CD42b and the MFI of CD42b on CD14+ cells.

Statistical analysis

Differences in patient characteristics across the ART-regime groups were compared using ANOVA with post-tests or chi-squared tests for proportions. Data of platelet reactivity are expressed as means with standard deviation (SD). PMA is expressed as median with interquartile range (IQR). Differences between groups were analysed with Student t-tests (platelet reactivity data) or with Mann-Whitney U test (PMA data). Subsequently, platelet reactivity data were analyzed in a general linear model with P-selectin expression and fibrinogen binding as dependent variables, ART group and duration of treatment as fixed factors and age, hypertension, and statins and abacavir use as covariates. All analyses were performed with SPSS version 20 (SPSS, Inc., Chicago, Illinois). $P<0.05$ were considered statistically significant.

Table 1. Characteristics of study population

Figure 1A-B shows the results of the platelet reactivity assay. A significantly lower P-selectin expression was observed in RAL-treated patients compared with NNRTI- and PI-treated patients, using both the low (P=0.026 vs. NNRTI and P=0.005 vs. PI-group) and high dose ADP concentration (P=0.009 vs. NNRTI and P=0.003 vs. PI-group). A similar trend for was found for fibrinogen binding. The RAL-treated group had lower fibrinogen binding to platelets, although this difference was only significant for the comparison with the PI-group after high dose ADP (P=0.038). P-selectin expression

	RAL	NNRTI	PI	Controls
Number	25	30	25	30
Male, n (%)	18 (72)	26 (87)	20 (80)	24 (80)
Age, years	50 (11)*	47 (11)	42 (9)	41 (9)
CD4 count, x109/L	667 (300)	666 (207)	721 (244)	NA
Cholesterol, mmol/L	4.8 (0.8)	4.7 (0.7)	4.7 (0.8)	ND
Platelets, x10^9/L	212 (35)	218 (58)	217 (36)	ND
Abacavir containing-regime, n (%)	6 (24)	9 (30)	3 (12)	NA
Hypertension history, n (%)	3 (12)	4 (13)	1 (4)	NA
Diabetes mellitus history, n (%)	1 (4)	4 (13)	2 (8)	NA
Statin use, n (%)	5 (20)	7 (23)	1 (4)	NA
Duration of ART use				NA
4-6 months, n (%)	3 (12)	5 (17)	0 (0)	
7-12 months, n (%)	1 (4)	6 (20)	1 (3)	
> 12 months, n (%)	21 (84)	19 (63)	24 (80)	

Data depicted are mean with SD unless otherwise indicated. Statistical differences are analyzed by using ANOVA with post-tests or chi-squared test. * P<0.05 versus PI and control group. RAL, raltegravir, NNRTI, non-nuclease reverse transcriptase inhibitor, PI, protease inhibitor, NA, not applicable, ND, not determined.

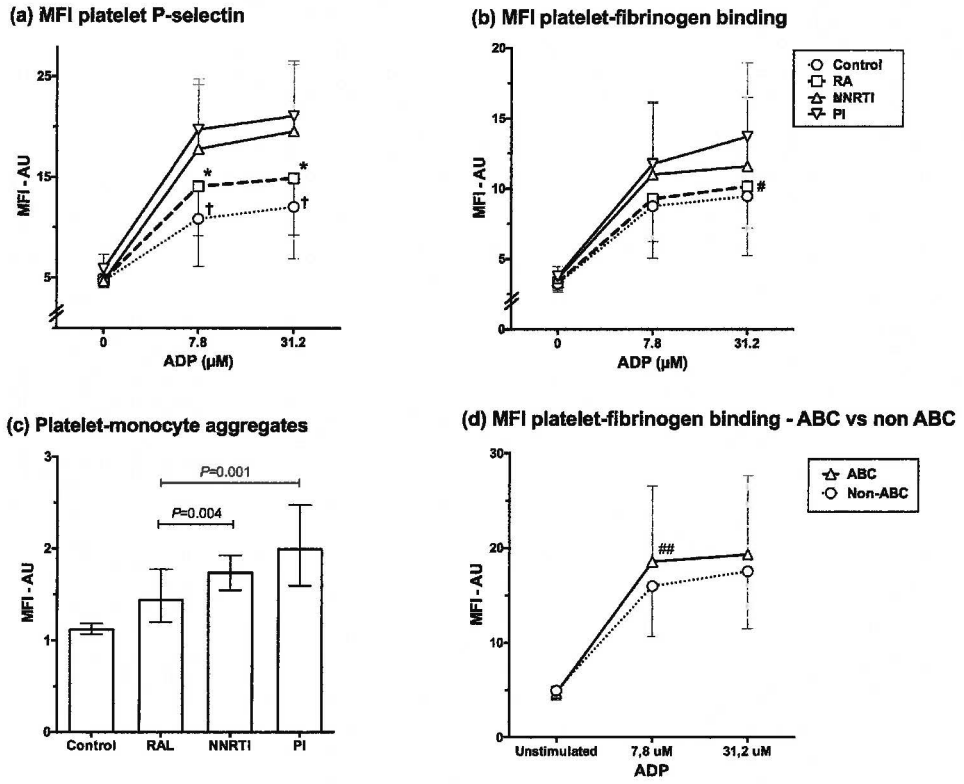

Figure 1. Platelet reactivity and platelet-monocyte aggregation in HIV1-infected individuals using different antiretroviral regimens.

(a) Platelet P-selectin expression and (b) fibrinogen binding to platelets, depicted as the mean fluorescence intensity (MFI) in arbitrary units (AU), at baseline and after stimulation with 2 concentrations of ADP in HIV1-infected individuals using a raltegravir (RAL)-based (n=25), non-nucleoside reverse transcriptase inhibitor (NNRTI)-based (n=30) or protease inhibitor (PI)-based (n=30) regimen. (c) Platelet-monocyte aggregation is depicted as the MFI of the platelet marker CD42b on CD14 positive cells. (d) Fibrinogen binding to platelets is shown in individuals using an abacavir (ABC) vs. non-ABC containing regimen. Data depicted are means with SD (platelet reactivity) or median with IQR (PMA). * $P<0.05$ RAL versus the NNRTI- and PI-treated groups; † $P<0.05$ control versus RAL group; # $P<0.05$ versus the PI group; ## $P<0.05$ versus the non-ABC group.

Results

A total of 80 HIV-infected patients were enrolled with a minimum of 25 patients per ART group and 30 individuals as healthy controls. Characteristics of the study participants are presented in Table 1. The groups were well matched for all characteristics except for age, which was significantly higher in the RAL group compared with the PI- and control group.

remained significantly higher in the RAL-treated group compared to healthy volunteers (P=0.011 and P=0.026 for low and high dose ADP, respectively). In unstimulated samples, the RAL group and the controls had a significantly lower mean (±SD) baseline P-selectin expression and platelet-fibrinogen binding compared to the PI group (MFI P-selectin: 4.8±0.8 and 4.7±0.5 vs. 5.9±1.5; P<0.005 and MFI fibrinogen binding: 3.3±0.5 and 3.2±0.6 vs. 3.8±0.7; P<0.02).

P-selectin mediates binding of platelets to monocytes (24). The RAL-treated group had a lower PMA compared to the NNRTI (P=0.004) and PI (P=0.001) groups, as indicated by a lower MFI of the platelet identification marker CD42b on CD14 positive cells (Figure 1C).

The NRTI abacavir has previously been shown to increase platelet reactivity (25). Abacavir was used by 17 (19.8%) of the HIV-infected participants in our study. Abacavir-treated individuals had a trend for increased platelet reactivity with higher P-selectin expression and fibrinogen binding (Figure 1D, only fibrinogen binding shown). The only significant difference was fibrinogen binding to the low ADP concentration (P=0.02). There were no significant differences in PMA between abacavir and non-abacavir treated individuals (data not shown). Finally, in a general linear model adjusting for duration of ART treatment, age, hypertension and the use of statins and abacavir, P-selectin expression in the RAL-treated group remained significantly lower compared to the NNRTI- (P=0.002 and P=0.003 for low and high dose ADP) and PI-treated group (P=0.001 and P=0.002, respectively).

Discussion

Our study shows that HIV-infected individuals on a RAL-based regimen show reduced platelet hyperreactivity and PMA compared with those on a NNRTI- or PI-based regimen. Our results also supports findings from previous studies that persisting platelet hyperreactivity and increased PMA are found in HIV-infected patients despite ART use (15-19), although reduced platelet aggregation by impedance aggregometry was also recently reported in both treated and untreated HIV-infected individuals (26).

Platelets play a pivotal role in atherosclerosis and acute cardiovascular events (5) and persisting platelet hyperreactivity may explain some of the excess risk for cardiovascular events in HIV beyond that explained by traditional cardiovascular risk factors (27). Our finding that a RAL-based regimen was associated with reduced platelet hyperreactivity and PMA may therefore be clinically relevant as higher values of these parameters are associated with an increased risk for future cardiovascular events (9, 12, 28-30).

Increased expression of both P-selectin, which mediates the interaction of platelets with monocytes, and the active conformation of $\alpha_{IIb}\beta_3$, which allows platelets to aggregate and

adhere via fibrinogen, which were both determined in our study, have been previously related to faster progression of atherosclerosis (7, 31, 32).

The mechanism underlying the lower platelet reactivity to ADP and PMA in RAL-treated individuals is still unexplained. The switch to a RAL-based regimen was associated with a decrease in inflammatory and coagulation markers in some studies (20, 21, 33) and reduced inflammation may be responsible for the lower platelet reactivity. Vice versa, the reduction in PMA and platelet hyperreactivity may also contribute to the decrease in inflammation in itself, as PMA and platelet activation induce a more proinflammatory status. Other possible mechanisms include the near absence of metabolic consequences of RAL (34) and its better control of residual HIV replication (35, 36) which may also influence platelet function as platelets have been shown to endocytose HIV virions (37). Our findings that the abacavir-treated individuals had a trend for higher platelet reactivity are consistent with those reported by Satchell (25). The confounding effect of abacavir on our results seems limited, as only two patients in the PI group and a comparable proportion of patients in the NNRTI and RAL groups used an abacavir-containing regimen.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, have been shown to inhibit platelet activation *in vivo* (38, 39) as well as having an anti-inflammatory effect (40). However, only a minority of participants in our study used a statin, limiting the possible confounding effects of statin use on our results. The fact that fewer patients in the PI group used a statin, despite the fact that mean cholesterol level across the groups were similar and PI's are associated with dyslipidemia, is probably explained by the choice of non-PI regimens in those with hyperlipidemia.

Whether reduced platelet reactivity and PMA in RAL-treated individuals are indeed associated with reduced cardiovascular complications requires studies with longer follow-up periods. Due to its near absence of adverse effects on glucose and lipid metabolism (34), RAL is among the preferred ART in patients with a high cardiovascular risk. Despite needing further clinical study, our findings provide an extra argument to consider a RAL-based regimen in these high risk patients. Our data also strengthen the case for preventive use of platelet function inhibitors in high risk patients. Aspirin was recently shown to attenuate platelet activation and immune activation in ART-treated patients in a small study (41). The increased platelet reactivity to ADP in our study supports the use of $P2Y_{12}$ receptor antagonists, also because these have been shown to reduce PMA (42). Finally, platelet function assays are currently not routinely used to identify patients with high platelet reactivity, but this may change with the availability of easy to use and sensitive assays as used in our study.

Limitations of our study are the fact that we used only one platelet agonist. However, previous studies performed by our group showed that platelet hyperreactivity to ADP is also associated with hyperreactivity to other agonists such as collagen or thrombin

receptor activating peptide (TRAP) (43). Secondly, due to the cross sectional nature of our study, causality cannot be proven. This will require a longitudinal, randomized study in which patients on a NNRTI- or PI-based regimen are switched to RAL.
In conclusion, we demonstrated lower platelet hyperreactivity and PMA in RAL-treated individuals. These observations warrant a larger randomized study in which the clinical benefits of a switch from other ARTs to a RAL-based regimen can be further investigated.

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Chapter 10

Platelet dysfunction contributes to bleeding complications in human leptospirosis

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Submitted.

Abstract

Background: Severe leptospirosis is frequently complicated by a hemorrhagic diathesis, of which the pathogenesis is still largely unknown. Thrombocytopenia is common, but often not to the degree that spontaneous bleeding is expected. We hypothesized that the hemorrhagic complications are not only related to thrombocytopenia, but also to platelet dysfunction, and that increased binding of von Willebrand factor (VWF) to platelets is involved in both platelet dysfunction and increased platelet clearance.

Methodology/Principle findings: A prospective study was carried out in Semarang, Indonesia, enrolling 33 hospitalized patients with leptospirosis, of whom 15 developed clinical bleeding, and 25 healthy controls. Platelet activation and reactivity were determined using flow cytometry by measuring the expression of P-selectin and activation of the αIIbβ3 integrin by the binding of fibrinogen in unstimulated samples and after *ex vivo* stimulation by the platelet agonists adenosine-diphosphate (ADP) and thrombin-receptor activating peptide (TRAP). Platelet expression of VWF, before and after VWF stimulation by ristocetin, as well as plasma levels of VWF, active VWF, the VWF-inactivating enzyme ADAMTS13, thrombin antithrombin complexes (TAT) and P-selectin were also measured. Bleeding complications were graded using the WHO bleeding scale. Platelets of the leptospirosis patients were activated with increased P-selectin and fibrinogen expression. In contrast, upon *ex vivo* platelet stimulation, P-selectin and fibrinogen expression were lower compared with healthy controls, which is indicative of platelet dysfunction. Patients with clinical bleeding had more pronounced platelet dysfunction than patients without bleeding and there was a significant inverse correlation of bleeding score with TRAP-stimulated P-selectin and fibrinogen expression (R=-0.72; P=0.003 and R=-0.46; P=0.034, respectively), but not with platelet count. Patients with bleeding also had a significantly higher platelet-VWF binding. Platelet counts were inversely correlated with platelet-VWF binding (R=-0.74; *P*=0.0009), but not with TAT levels.

Conclusion/Significance: Platelet dysfunction is common in leptospirosis patients with manifest bleeding. Increased VWF-platelet binding may contribute to activation and clearance of platelets. Our data suggest that platelet transfusion or possibly novel anti-VWF therapies may have clinical benefit in leptospirosis patients with bleeding.

Introduction

Leptospirosis is a zoonotic disease of global importance caused by the pathogenic spirochaetes of the genus *Leptospira* [1, 2]. A modeling exercise by the World Health Organization's (WHO's) Leptospirosis Burden Epidemiology Group, estimated that 873,000 cases and 48,600 deaths occur worldwide each year [3]. The clinical manifestations of leptospirosis range from a mild, self-limited febrile illness to a fulminant life-threatening illness with multi-organ failure [1, 2]. Bleeding complications are common in severe leptospirosis, being reported in up to 60% of all hospitalized patients. Although the majority of bleeding events are mild, some patients may develop severe gastrointestinal or pulmonary haemorrhage, the latter having an alarmingly high mortality of >50% [4, 5].

The pathophysiological mechanisms responsible for bleeding remain incompletely understood. Thrombocytopenia is frequently observed and is associated with poor outcome, [6] but its severity is not to the extent that spontaneous bleeding is expected [7]. Platelet dysfunction might also contribute to bleeding. Measuring platelet function in thrombocytopenic conditions is technically demanding as commonly used techniques such as light transmission aggregometry are not useful [8]. To the best of our knowledge, no studies on platelet function in leptospirosis have been performed.

The binding of von Willebrand factor (VWF) to the glycoprotein (GP)-1b receptor on platelets is a well-known platelet clearance mechanism [9]. VWF is a multimeric glycoprotein that predominantly originates from the endothelium. Its main function is the recruitment of platelets to sites of vascular injury. Under normal conditions, most of the VWF circulates in non-platelet binding form. VWF may however undergo a conformational change to a more active, platelet-binding form facilitating platelet aggregation [10]. An increase in this so-called 'active' VWF has been described in a number of infectious and non-infectious diseases [11-13]. A deficiency in ADAMTS13 (a disintegrin and metalloproteinase thrombospondin type 1 motif, member 13), an enzyme that cleaves large VWF multimers into their inactive conformation, may also lead to excessive intravascular platelet agglutination [14]. Recently, it was shown that excessive VWF binding to platelets not only leads to thrombocytopenia, but also to thrombocytopathy in von Willebrand disease type 2B [15].

We hypothesized that thrombocytopenia in leptospirosis is associated with increased VWF-platelet binding and that the hemorrhagic complications are associated with thrombocytopathy due to excessive platelet activation and VWF-platelet binding. We therefore characterized VWF binding to platelets, platelet activation and platelet reactivity using flow cytometry in adult Indonesian patients with severe leptospirosis and determined whether these parameters were related to bleeding complications. In addition, we determined the plasma levels of VWF, active VWF, P-selectin, the plasmatic coagulation factor thrombin antithrombin complexes (TAT) and ADAMTS13 activity.

Methods

Study population

We carried out a prospective study at the Department of Internal Medicine of Dr. Kariadi Hospital, Semarang, Indonesia, between December 2013-March 2014. We enrolled adult patients admitted with a high clinical suspicion of leptospirosis using the case definition of the World Health Organization South East Asia Regional Office (WHO SEARO, 2009) and a positive result of rapid IgM lateral flow test, which had reported sensitivity and specificity of 85.6% and 96.2%, respectively [16]. Clinical diagnosis of leptospirosis was confirmed in a single acute sample by microscopic agglutination test (MAT) with a panel consisting of 31 serovars (28 pathogenic serovars: Australis, Bratislava, Autumnalis, Rachmati, Ballum, Castellonis, Bataviae, Benjamini, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Hardjo, Icterohaemorrhagiae, Lai, Naam, Coxi, Javanica, Panama, Pomona, Proechimys, Pyrogenes, Sarmin, Saxkoebing, Sejroe, Shermani, Tarassovi; three non-pathogenic serovars: Andamana, Patoc and Semaranga). A titre of ≥1/320 on a single sample was considered positive. All diagnostic tests were performed at the National Reference Laboratory for Leptospira, Dr. Kariadi Hospital, Semarang, Indonesia. Patients and healthy controls were included after written informed consent was obtained. Blood samples were obtained on inclusion day (day 1) and upon follow up (day 4). The study is approved by the ethical committee of the Faculty of Medicine Diponegoro University-Dr. Kariadi Hospital, Semarang, Indonesia.

Platelet activation and reactivity and platelet-von Willebrand factor binding

Whole blood was collected from patients by using venipuncture from the antecubital vein into citrate-anticoagulated tubes (3.2%; BD Vacutainer, Becton Dickinson). All samples were processed within one hour after blood collection. Platelet activation and reactivity were determined by flow cytometry using a method previously described [17, 18]. In short, the platelet membrane expression of P-selectin (CD62P) and platelet-fibrinogen binding, which correspond with platelet degranulation and aggregation, were determined in unstimulated whole blood and in whole blood stimulated 20 min with the platelet agonists adenosine-diphosphate (ADP; 7.8 μM and 31.2 μM) or thrombin receptor-activating peptide (TRAP; 39 μM and 625 μM) diluted in HEPES-buffered saline. Saturating concentrations of the following monoclonal antibodies were used: PE-labeled anti-CD62P (Bio-Legend, San Diego, USA), FITC-labeled anti-fibrinogen (DAKO Ltd., High Wycombe, UK), and PC7-labeled anti-CD61 (platelet identification marker; Beckman Coulter, France). Platelets were gated based on their forward- and sideward-scatter (FSC/SSC) properties and positivity for CD61, which was defined as a median fluorescence intensity (MFI) exceeding the MFI of the matched isotype control.

The binding of vWF to platelets was determined by adding whole blood sample to a mixture of HEPES-buffered saline and saturating concentrations of FITC-labeled anti-von Willebrand factor (Abcam, Cambridge, UK) and PC7-labeled anti-CD61, with or without the addition of ristocetin (0,84 mg/ml and 1,5 mg/ml). After incubation for 20 minutes at room temperature, a fixative solution (0.2% paraformaldehyde) was added and samples were analyzed with a BD FACS Canto II flow cytometer (Becton Dickinson, USA). Next, the MFI of CD62P, fibrinogen and VWF on CD61-positive events were determined.

Plasma markers

Platelet-poor plasma was obtained from citrate-anticoagulated whole blood by centrifugation (1500 g without brake, 15 min, 20°C). Plasma concentrations of thrombin-antithrombin (TAT) complexes were subsequently measured with enzyme-linked immunosorbent assay (ELISA) as previously described [24]. Sheep anti-human thrombin (SAHT-AP, SAHT-HRP) antibodies were purchased from Kordia/Affinity Biologicals, USA. Plasma VWF concentrations were determined with ELISA as described previously [19]. Active VWF was quantified by ELISA using a nanobody (AU/VWFa-11) that recognizes the GPIb binding configuration of VWF, as described previously [20]. We express the relative amount of VWF that circulates in its active, platelet binding conformation by using the term VWF activation factor. VWF activation factor of normal pooled plasma was referred to as 1. ADAMTS13 activity was quantified using the fluorescence resonance energy transfer (FRETS) assay (Peptides International, Lexington, USA), whereby the ADAMTS13 activity of normal pool plasma was set at 100%, and the values obtained in study participant samples were expressed as percentage of normal pool plasma [21].

Statistical analysis

Differences in subject characteristics were compared with analysis of variance (ANOVA) with posttests for comparing more than two groups, and with Mann-Whitney U-test or chi-square test for comparing the two patient groups. Data are presented as median with interquartile range (IQR) unless stated otherwise. Relationships between parameters were assessed with the Spearman correlation coefficient. All analyses were performed with SPSS version 20 (SPSS, Inc., Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 33 consecutive hospitalized leptospirosis patients with a positive rapid IgM-based, lateral flow serology were enrolled together with 25 healthy controls. Twelve (36%) of these patients had a positive result of the MAT (titer >1/320). Characteristics of participants are presented in Table 1. Fifteen patients (45.5%) presented with or developed clinical relevant bleeding manifestations during hospitalization. The most common were gastrointestinal (n=12) and genitourinary (n=8) bleeding events. The severity of bleeding events in patients were graded using the WHO bleeding scale, which has been used extensively worldwide [22-24]. Ten out of 15 patients who experienced clinical bleeding developed Grade 2 bleeding (i.e. epistaxis with a total duration of all episodes in previous 24 hours of >30 minutes, grossly visible blood in urine, stool or emesis), while 4 developed that of Grade 1 (i.e. epistaxis with a total duration in previous 24 hours of <30 minutes, microscopic hematuria and petechiae). There was 1 patient with grade 3 bleeding whose haematemesis and melena caused haemodynamic instability. One grade 1 and three grade 2 patients died during hospitalization.

Table 1.

	All patients	Bleeders	Non-bleeders	Reference values	Controls	*P* value
Numbers (%)	33	15 (46)	18 (54)		25	
Male sex, n (%)	22 (67)	7 (46.7)	15 (83)		17 (68)	0.03
Age (years)	47 (36-53)	52 (38-59)	48 (40-52)		36 (32-50)	0.24
Bleeding events, n (%)						
Oral, nasal, skin						
Epistaxis		2 (6)				
Petechiae		2 (6)				
Gastrointestinal						
Hematochezia		1 (3)				
Hematemesis		5 (15)				
Melena		4 (12)				
Genitourinary						
Microscopic hematuria		3 (9)				
Gross hematuria		5 (15)				
Days post-onset illness, n	8 (7-11)	7 (6-10)	7 (7-12)			0.12
Hb (gr/dl)	13.3 (10.8-15)	10.9 (9.4-13.2)	14.8 (13.9-16.5)	12-15		0.004
Platelet count (x109/l)	114 (60-172)	105 (56-152)	123 (106-183)	150-300		0.003
Thrombocytopenia <150 x109/l (%)	17 (52)	10 (67)	7 (39)			0.11
Thrombocytopenia <100 x109/l (%)	9 (27)	7 (47)	2 (11)			0.03
Bilirubin (mg/dl)	1.6 (0.8-6.4)	2.0 (1.5-5.2)	1.3 (0.8-5.3)	0-1.0		0.28
AST (U/l)	89 (44-147)	84 (58-131)	95 (46-138)	15-35		0.72
ALT (U/l)	93 (63-160)	87 (62-131)	99 (79-193)	15-60		0.68
Ureum (mg/dl)	47 (31-196)	64 (46-221)	36 (24-49)	15-39		0.06
Creatinine (mg/dl)	1.7 (1.1-4.6)	2.0 (1.4-5.1)	1.4 (1-2)	0.6-1.3		0.12

Numbers represent median (IQR) values of 33 patients. *P* values represent difference between bleeders and non-bleeders (Mann-Whitney U-test or chi-square test when appropriate). A *P* value <0.05 was considered statistically significant.

Median (IQR) platelet count was significantly lower in the group of bleeders compared with the non-bleeders (105 (56-152) x10^9/l vs. 123 (106-183) x10^9/l; *P*=0.003). Five patients had a platelet count lower than 50 x10^9/l (bleeders: 12 x10^9/l, 17.4 x10^9/l, 29.7 x10^9/l, 30.0 x10^9/l; non-bleeder: 48.6 x10^9/l). Additionally, the haemoglobin levels were lower in the bleeders group than the non-bleeders group (10.9, 9.4-13.2 gr/dl vs. 14.8, 13.9-16.5 gr/dl; *P*=0.004), with six patients having haemoglobin levels below 10gr/dl (bleeders: 8.2 gr/dl, 8.9 gr/dl, 9.1 gr/dl, 9.4 gr/dl, 9.8 gr/dl; non-bleeder: 9.5 gr/dl).

Bleeding is associated with higher platelet activation with platelet dysfunction

Figure 1A shows the fibrinogen binding to the activated αIIbβ3 receptor and expression of P-selectin on platelets. Patients with bleeding had a significantly higher platelet-fibrinogen binding (MFI 2601, 1726-2938 vs. 1577, 1460-1752; *P*=0.001) and P-selectin (MFI 1095, 798-1685 vs. 654, 575-1056; *P*=0.002) in unstimulated blood samples than controls, suggesting increased platelet activation. The non-bleeding patients also had a significantly higher membrane P-selectin expression compared to controls (MFI 1179, 910-1444; *P*=0.003). Platelet reactivity was assessed by *ex vivo* stimulation of whole blood with two concentrations of the platelet agonists ADP or TRAP. In contrast to the findings in unstimulated samples, P-selectin expression and fibrinogen binding in response to TRAP or ADP were lower in the bleeders compared with non-bleeders and controls, suggestive of platelet dysfunction. A reduction in platelet reactivity, albeit to a lesser extent, was also found in the non-bleeders in response to TRAP.

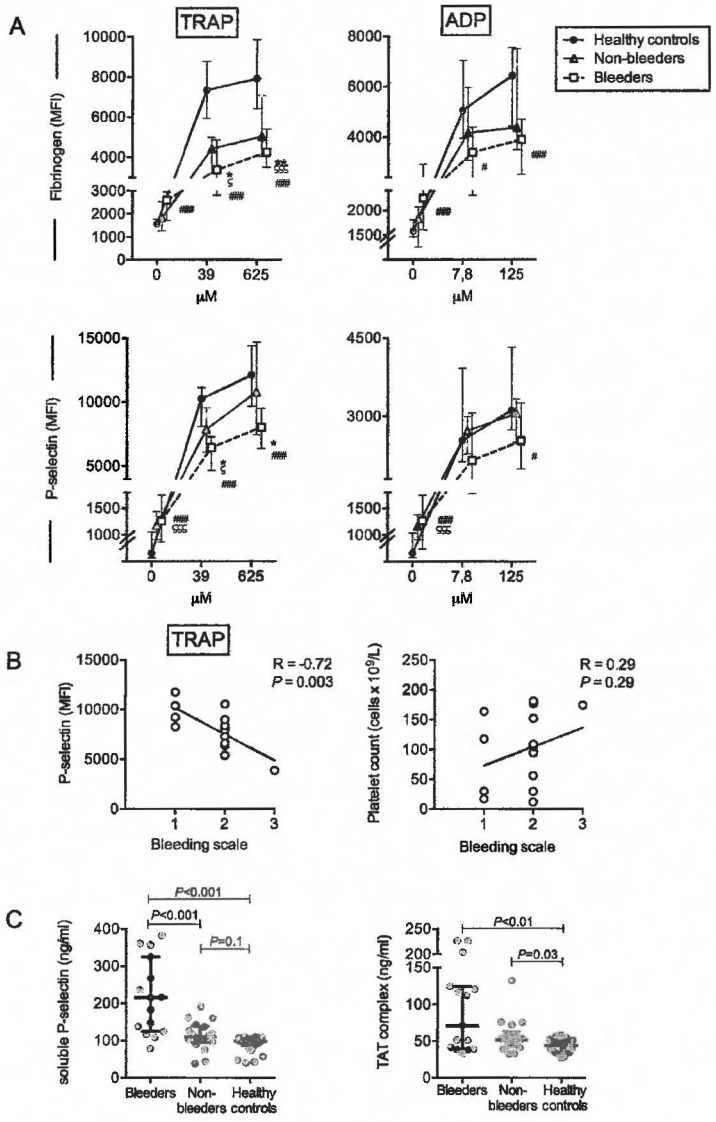

Figure 1. Platelet reactivity, soluble P-selectin and the relationship between bleeding and thrombocytopathy.

(A) Platelet-fibrinogen binding and the platelet membrane expression of P-selectin (depicted as median fluorescence intensity (MFI) in arbitrary units) in unstimulated samples and after stimulation with two concentrations of the platelet agonists thrombin receptor activating peptide (TRAP) and adenosine diphosphate (ADP) in leptospirosis patients with bleeding (bleeders, n=15) and without bleeding (non-bleeders, n=18) and in healthy controls (n=25). Data depicted are medians with IQR. * $P<0.05$ bleeders vs. non-bleeders, # $P<0.05$ bleeders vs. healthy controls, ### P<0.001 bleeders vs. healthy controls, ς $P<0.05$ non-bleeders vs. healthy controls, ςςς $P<0.001$ non-bleeders vs. healthy controls. (B) Correlation between the severity of bleeding, as measured using the WHO bleeding scale, and platelet P-selectin expression in response to TRAP (625 µM; left panel) as well as platelet count (right panel). Shown are the Spearman correlation coefficient. (C) Plasma concentration of soluble P-selectin and thrombin-antithrombin (TAT) complex. Data depicted are medians.

Interestingly, bleeding score was associated with impaired platelet P-selectin expression and platelet-fibrinogen binding in response to TRAP (R=-0.72; *P*=0.003 and R=-0.46; *P*=0.034, respectively) but not with platelet count (data for bleeding score correlation with P-selectin and platelet count shown in Fig 1B). The flow cytometric findings of platelet activation in bleeders were supported by measurement of plasma concentrations of soluble P-selectin, which was significantly higher in bleeders compared to both the non-bleeders and healthy controls (Fig 1C). Plasma P-selectin in the non-bleeders was comparable with that of the controls. Overall, leptospirosis patients also had significantly higher plasma TAT concentrations, suggesting activation of the plasmatic coagulation, with the highest concentrations found in the bleeders (Fig 1C).
Follow up data at day four were available in 11 bleeders and 10 non-bleeders. Bleeders, but not the non-bleeders, remained having a significantly reduced platelet reactivity in the follow up on day 4 compared to healthy controls. (Suppl. Fig 1A). Plasma P-selectin also remained significantly increased at day 4 in bleeders (Suppl. Fig 1B).

Platelet von-Willebrand factor binding was highest in bleeders
Binding of VWF to platelets is associated with platelet clearance, platelet activation and also possibly platelet dysfunction [15, 25]. We determined platelet-VWF binding using flow cytometry and measured plasma VWF levels, VWF activation factor and ADAMTS13 activity. Bleeders had a significantly higher VWF expression on circulating platelets (MFI 9069, 7470-10554) than non-bleeders (MFI 6999, 4707-8250; *P*=0.01) and healthy controls (MFI 7296, 6877-7867; *P*=0.002) (Fig 2A). Upon activation of VWF by ristocetin, the bleeding patients demonstrated the highest increase in VWF expression on platelets (Fig 2B). These flow cytometric findings were consistent with the observation that bleeders had the highest plasma VWF concentration (Fig 2C) and that the VWF activation factor was about twofold higher in both the bleeding and non-bleeding leptospirosis patients than in healthy controls, indicating that a higher amount of the circulating VWF was in an active, platelet-binding conformation (Fig 2D). Data on follow-up measurements on day 4 are presented in Suppl. Fig 2A-C.

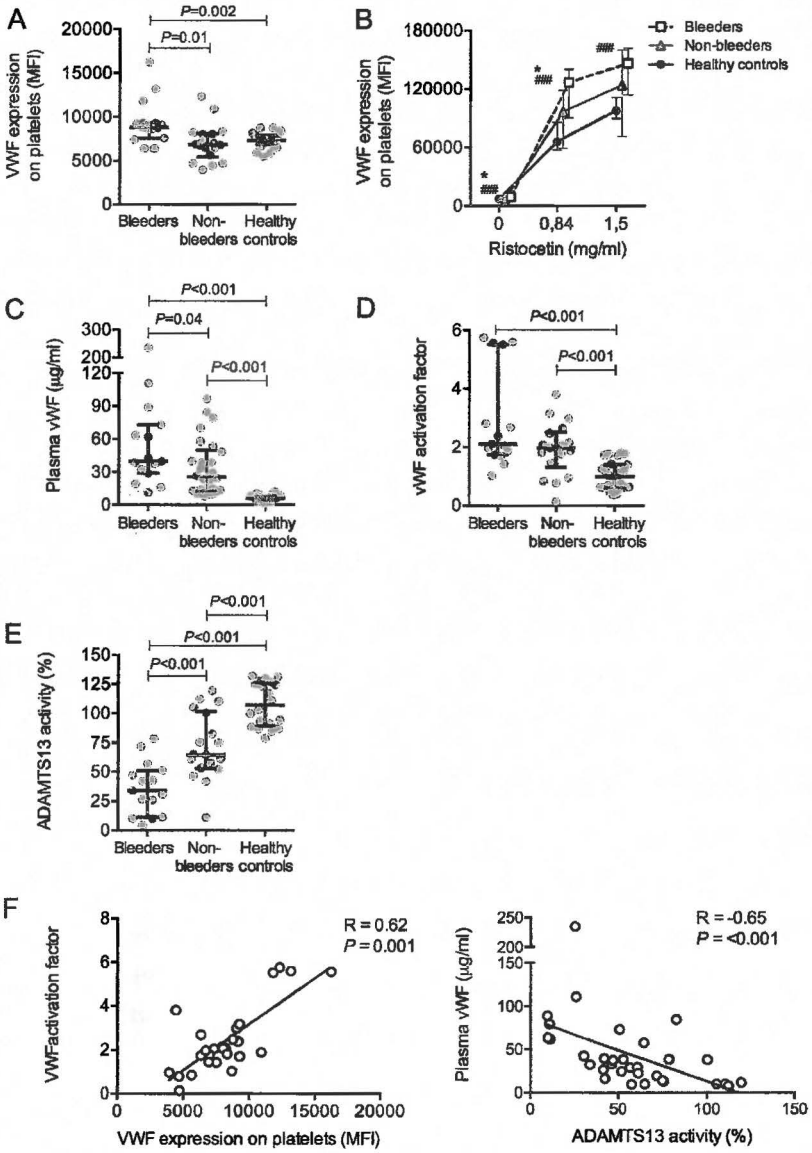

Figure 2. Platelet-von Willebrand factor (VWF) binding, plasma VWF, VWF activation factor and ADAMTS13 levels.

VWF expression on platelets (depicted as median fluorescence intensity (MFI) in arbitrary units) in unstimulated samples (A) and after *ex vivo* stimulation with two concentrations of ristocetin (B). *P<0.05 bleeders vs. non-bleeders, ### P<0.001 bleeders vs. healthy controls. (C-E) Plasma concentrations of VWF, VWF activation factor and ADAMTS13 activity. Data are shown as medians with IQR. (F) Spearman correlation coefficient of VWF activation factor and platelet-VWF binding (left panel), as well as plasma VWF and ADAMTS13 activity (right panel) of leptospirosis patients with and without bleeding.

ADAMTS13 functions as a natural regulator that de-activates the VWF by proteolysis [26] and can be consumed by high level of circulating VWF [12]. ADAMTS13 activity levels were decreased in leptospirosis patients and was lowest in bleeders (Fig 2E). Three patients in the bleeders group had ADAMTS13 activity level lower than 10%. An ADAMTS13 activity below 50% was found in 11 (73%) patients in the bleeders group and only in three (25%) patients in non-bleeders group. In the leptospirosis group as a whole, VWF expression on platelets correlated positively with the VWF activation factor (R=0.62; *P*=0.001). Although ADAMTS13 activity did not show any correlation with VWF ctivation factor, it had an inverse correlation (R=-0.65; *P*=0.0009) with plasma VWF levels, suggesting consumption of ADAMTS13 by VWF (Fig 2F).

Platelet-vWF binding is correlated with degree of thrombocytopenia

Next, we checked associations between parameters to explore possible mechanisms underlying the leptospirosis-associated thrombocytopenia and platelet dysfunction (Fig 3). First, there was a strong inverse correlation of platelet count with platelet-VWF binding and to a lesser extent with the platelet membrane expression of P-selectin (Fig 3A-B, day 4 data presented in Suppl. Fig 2D). In contrast, plasma level of the coagulation activation marker TAT complex did not correlate with platelet numbers. Finally, VWF-platelet binding was associated with fibrinogen expression in unstimulated samples and after TRAP and ADP stimulation (data from unstimulated and TRAP- stimulated fibrinogen expression shown in Fig 3D).

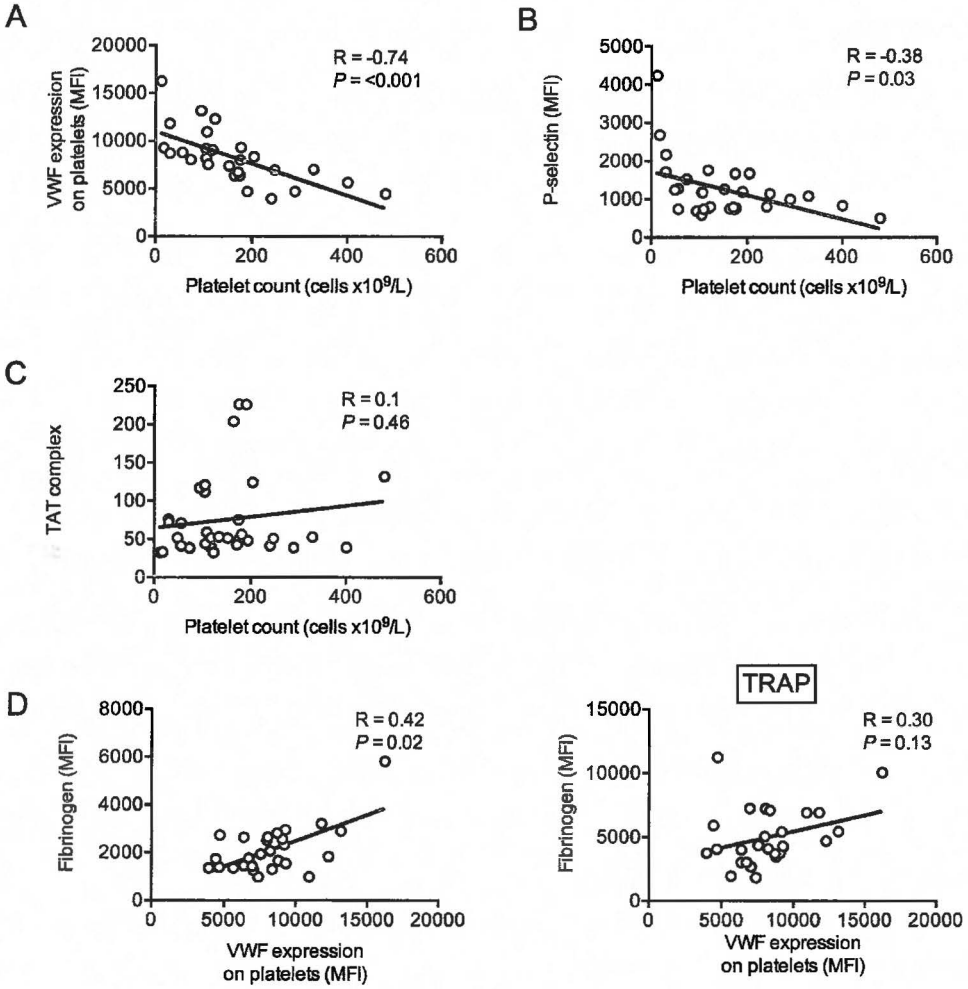

Figure 3. Platelet count and platelet-VWF binding correlates.
(A-C) Spearman correlation of platelet count with platelet-VWF binding, platelet P-selectin expression and thrombin anti-thrombin complex. (D) Spearman correlation of platelet-VWF binding and platelet-fibrinogen binding in unstimulated sample (left panel) and upon TRAP (625 μM) stimulation (right panel). Data for leptospirosis patients with and without bleeding are shown.

Discussion

Our study reveals four important findings. First, platelet activation is a feature of leptospirosis with bleeding manifestations. Second, circulating platelets in leptospirosis patients express more VWF and this has a strong negative association with platelet number, in contrast with the plasmatic coagulation marker TAT complex. Third, VWF binding to platelets was associated with platelet activation, but not with platelet dysfunction, as recently reported for von Willebrand disease type 2B [15]. Four, bleeding complications are predominantly associated with platelet dysfunction rather than absolute platelet count.

To the best of our knowledge, this is the first study measuring *in vivo* platelet activation and reactivity in human leptospirosis. Platelet function studies are logistically challenging, as blood samples need to be processed without delay. In conditions with thrombocytopenia, aggregometry is also less reliable and flow cytometry-based assays are preferred [8]. To our knowledge, our study is the first to report results from platelet function assays in leptospirosis. Our findings are in line with earlier observations that activated platelets are present in the lung capillaries of patients who died of leptospirosis [29] and in hepatic sinusoids from guinea pigs experimentally infected with leptospirosis [30]. The latter study also showed that thrombocytopenia was not related to disseminated intravascular coagulation, as is also suggested by our findings showing an absent correlation of TAT complex levels with platelet count. Another animal study, using a virulent serovar of *Leptospira interrogans* in gerbils, reported increased levels of platelet-activating factor acetylhydrolase (PAF-AH), which might contribute to inhibition of platelet activation [31]. Our observation that platelet function, rather than the absolute platelet count, determined the bleeding risk also does not stand alone. Increasing evidence from patients with immune thrombocytopenic purpura (ITP) has identified platelet function as an important determinant of bleeding risk [32-34].

High plasma VWF levels, together with elevations in other endothelial cell activation markers, were recently reported in patients with leptospirosis [35]. Our findings add to this by showing that the circulating VWF is in an active, GPIbα-binding conformation, and that circulating platelets indeed have more VWF on their surface. Most VWF is derived from endothelial cells. However, platelets also contain VWF in their granules [36] and to what extent this contributes to the increased VWF expression on the platelet membrane is unknown. We also found a concurrent decrease in ADAMTS13 activity levels. This enzyme regulates the multimeric size and function of VWF through the cleavage of VWF within the A2 domain. A severe reduction in ADAMTS13 activity as a result of auto-antibodies are a hallmark of the rare disease thrombotic thrombocytopenic purpura (TTP) [37]. Indeed, severe leptospirosis shows some similarities with TTP with thrombocytopenia and organ failure. Cases of leptospirosis-associated TTP have also been

described, including that with severely reduced ADAMTS13 activity [38, 39]. Infections may lead to a significant reductions in ADAMTS13 as a results of different mechanisms, as recently reviewed by Schwameis [40]. Multiple studies have shown that conditions with increased VWF release are associated with secondary ADAMTS13 consumption, such as in severe systemic infections [27] and after desmopressin-induced VWF release [28]. Inhibition of ADAMTS13 activity can also occur due to inflammation-induced IL-6 release [41] or proteolytic cleavage of ADAMTS13 by neutrophils [42]. And lastly, competition of ADAMTS13 with thrombospondin-1 for the interaction with VWF-A3 domain may slow the proteolysis of UL-VWF multimers [40].

The mechanisms underlying the observed platelet activation and platelet dysfunction in our study population remain to be elucidated. It is unknown whether pathogenic *Leptospira interrogans* strains are able to directly interact with and activate platelets. Leptospiral lipopolysaccharide (LPS) was shown to be a ligand of Toll-like receptor (TLR)-2 in humans and of TLR-4 and TLR-2 in mice [43]. Platelets harbor both TLRs and especially ligation of TLR-2 leads to a strong thrombotic platelet activating response [44]. Alternatively, increased platelet-VWF binding may activate platelets [45]. In patients with von Willebrand disease type 2B (VWD type 2B), a diseases characterized by gain-of-function mutations in VWF that enhances its spontaneous binding to the platelet GPIbα, increased platelet-VWF binding is associated with thrombocytopathy due to inhibition of αIIbβ3 activation [15]. However, platelet-VWF binding was not correlated inversely with fibrinogen binding to the activated αIIbβ3 receptor in response to platelet agonists, rendering this as a less likely cause for thrombocytopathy in leptospirosis. Rather, we speculate that excessive platelet activation with secondary platelet 'exhaustion' underlies the observed platelet dysfunction. We previously found a similar phenotype in patients with dengue [46].

In contrast, we did find a strong inverse correlation of VWF-platelet binding and platelet number, suggesting that VWF expression on the platelet membrane is involved in platelet clearance. Increased platelet-VWF interaction in VWD type 2B results in increased platelet clearance by the liver [47]. How the VWF-platelet complexes are being cleared remains to be determined. Evidence suggests that glycans on GPIbα are critical in mediating platelet clearance via receptors containing carbohydrate-binding domains on the macrophage αMβ2 integrin and the hepatic Ashwell-Morell receptor [25, 48, 49]. One plausible mechanistic explanation is that the increased platelet-VWF binding results in a structural unfolding of the GPIbα extracellular domain and triggers signaling into the platelet, desialylation of the platelet surface and platelet clearance [50].

The limitations of our study include the small number of patients tested positive with the MAT and the fact that patients were included based on rapid IgM seropositivity and clinical manifestations consistent with leptospirosis. Regardless, the use of MAT as

a gold standard of leptospirosis diagnosis in the real clinical setting has been criticized for its low sensitivity of 70% even in the ideal situation, in which convalescent-phase samples were obtained from all patients [16]. Our study was also performed in Semarang, Indonesia, where leptospirosis is a common cause of undifferentiated fever [51].

In conclusion, we found that platelet activation and platelet dysfunction are features of leptospirosis, which are associated with the severity of bleeding events. Circulation platelets also express more VWF, and although this does not explain the observed platelet dysfunction, it may play a role in enhanced platelet clearance. Bleeding remains as a serious, life-threatening complication of leptospirosis and our findings warrant further study on the clinical utility of platelet function test, as it is thrombocytopathy rather than thrombocytopenia that is associated with the severity of bleeding events. In severe bleeding, platelet transfusion may temporarily reverse the platelet dysfunction. Potential future therapeutics can be directed at inhibiting platelet-VWF binding, for example by using an anti–VWF humanized single-variable-domain immunoglobulin, caplacizumab to inhibit the GPIb binding site of VWF [52] or an antagonist of the platelet GPIb receptor, anfibatide [53]. Alternatively, administration of recombinant ADAMTS13 might have a therapeutic role in those with severely reduced ADAMTS13 activity [54].

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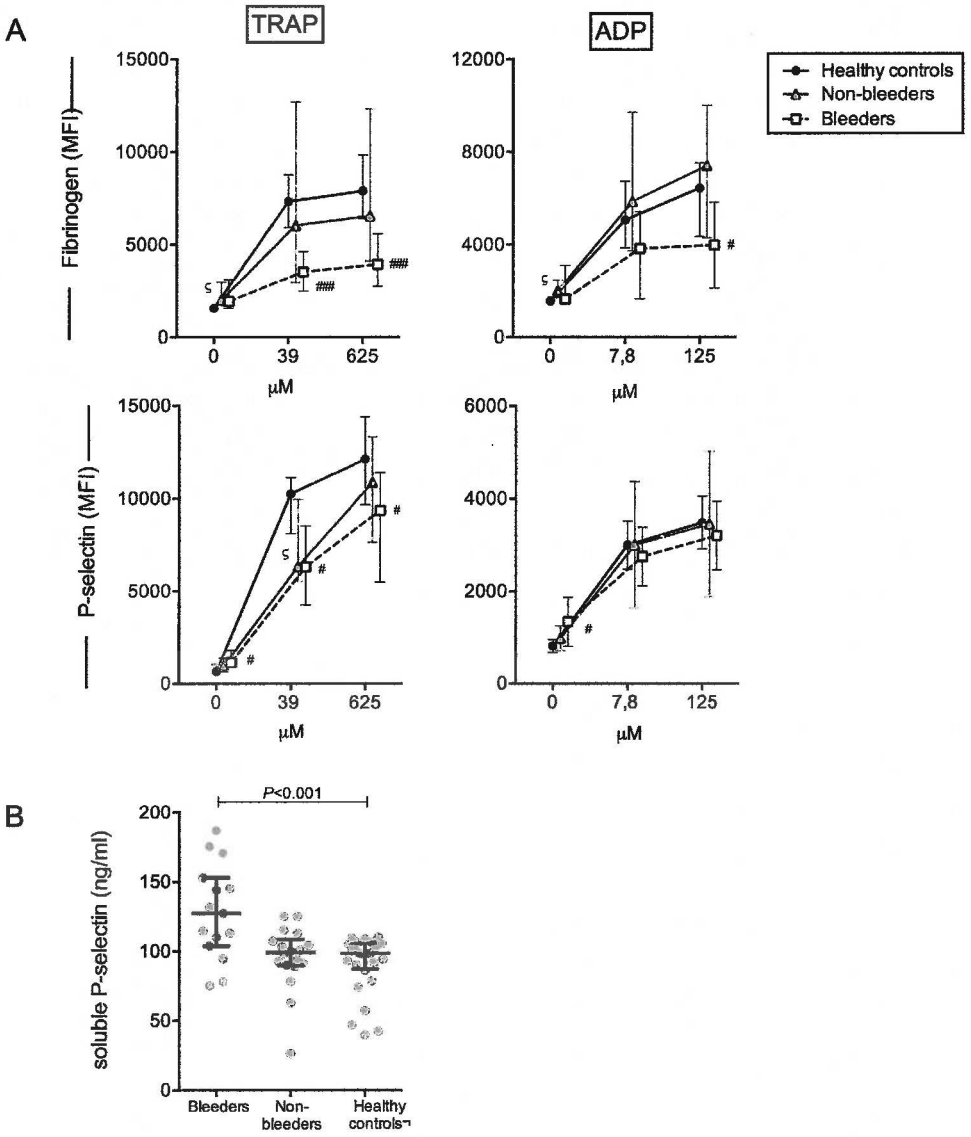
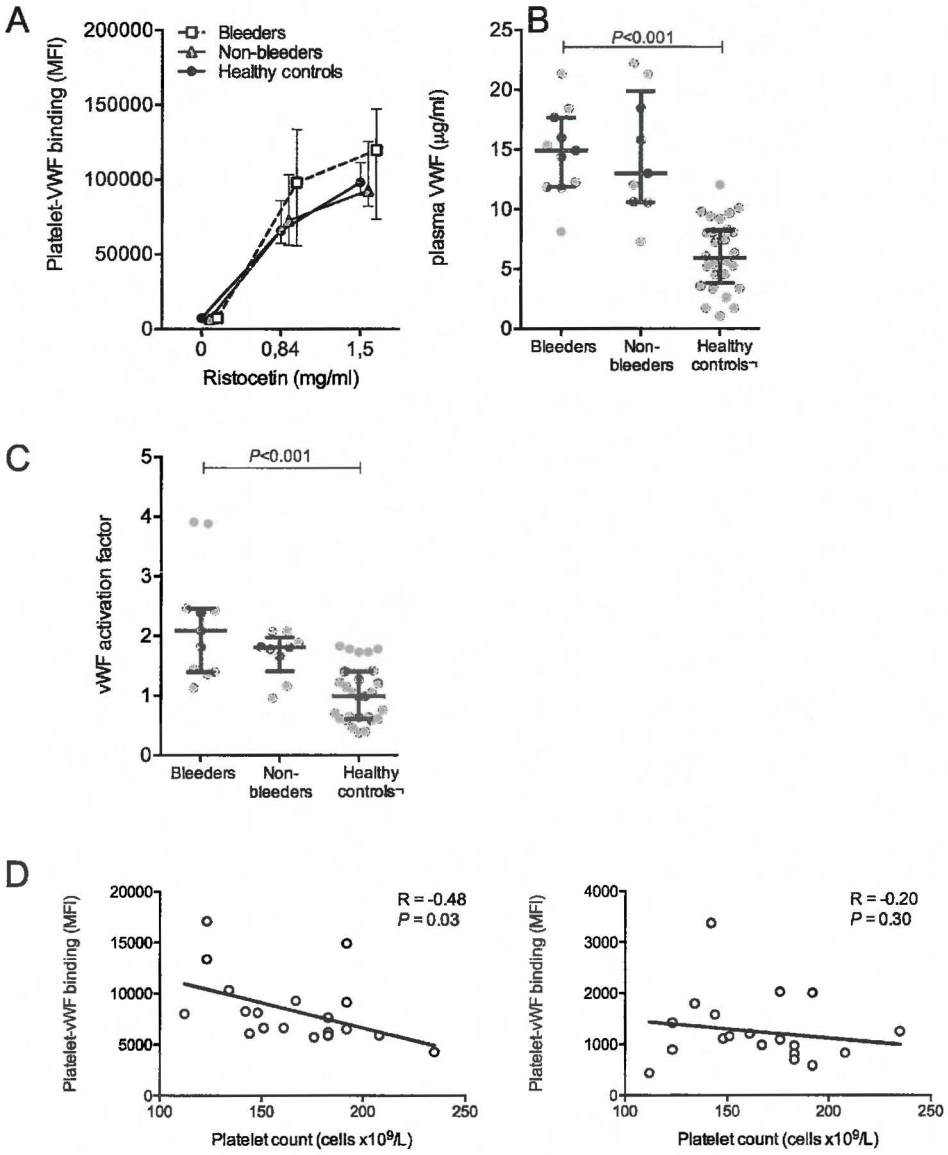
Supplemental Figures



Suppl. Fig 1. Platelet reactivity and soluble P-selectin upon follow up.
(A) Platelet-fibrinogen binding and the platelet membrane expression of P-selectin (depicted as median fluorescence intensity (MFI) in arbitrary units) in unstimulated samples and after stimulation with two concentrations of the platelet agonists thrombin receptor activating peptide (TRAP) and adenosine diphosphate (ADP) in leptospirosis patients with bleeding (bleeders, n=15) and without bleeding (non-bleeders, n=18) and in healthy controls (n=25). * $P<0.05$ bleeders vs. non-bleeders, # $P<0.05$ bleeders vs. healthy controls, ς $P<0.05$ non-bleeders vs. healthy controls. (B) Plasma concentration of soluble P-selectin. Data depicted are medians with IQR. Data of patients were from day 4, while data from healthy controls were from day 1.



Suppl. Fig 2. Platelet-von Willebrand factor (VWF) binding, plasma VWF, VWF activation factor and platelet count correlates upon follow up.

(A) VWF expression on platelets (depicted as median fluorescence intensity (MFI) in arbitrary units) in unstimulated samples and after *ex vivo* stimulation with two concentrations of ristocetin. (B-C) Plasma concentrations of VWF and VWF activation factor. (D) Spearman correlation coefficient of platelet count and platelet-VWF binding as well as platelet P-selectin expression. Data are shown as medians with IQR. Data from patients were from day 4, while data from healthy controls were from day 1.

Chapter 11

General Discussion

General Discussion

The first part of this doctoral thesis focuses on the interactions of platelets with inflammation and immunity. Studies were predominantly performed in healthy volunteers and included the interlinking of platelet reactivity and inflammatory responses at the functional and genetic levels (chapter 2) and the differential effects of platelets on Toll-like Receptor (TLR)-2 and TLR-4-induced immune responses (chapter 3). In addition, we described the platelet-inhibiting action of recombinant alkaline phosphatase (recAP) and its mechanisms of action (chapter 4) and studied the effects of hypoxia (chapter 5) on platelet function in the context of systemic inflammation.
The second part of this thesis focuses on the alterations of platelet function due to infections. Studies were performed in an animal model and in patients with infectious diseases. We investigated changes in platelet activation associated with pneumococcal bacteremia (chapter 6), a phage-derived gene of *Streptococcus pneumoniae* (chapter 7), Gram-positive/Gram-negative bacteremia (chapter 8) and different treatment regimens of HIV-infected individuals (chapter 9). We also investigated changes in platelet function in leptospirosis patients and related this to bleeding complications (chapter 10).

The interaction between platelets and inflammation

Platelets alter inflammatory and immune responses
Platelets are known to play a key role in inflammation and host defense. Platelet function is therefore likely to affect immune responses and the pathogenesis of certain infectious diseases. The opposite also occurs: inflammation and infections significantly affect platelet numbers and function. The extent and mechanisms of these interactions remain to be fully elucidated. Most available data originate from *in vitro* or animal studies (reviewed in (1-3)), and from clinical studies in patients with infections (4-6) or chronic inflammation (7-9). At the genetic level, studies have identified quantitative trait loci (QTLs) regulating platelet function (reviewed in (10)) or immune responses (11, 12) but have not interlinked these two processes. In **chapter 2**, we performed platelet reactivity assays and measurements of cytokine responses of whole blood and peripheral blood mononuclear cells (PBMCs) in the 500-Human Functional Genomics (500FG) cohort of approximately 500 Caucasian, healthy individuals as part of the Human Functional Genomics Project (HFGP). We assessed the associations between platelet number and reactivity as well as cytokine responses. We found that platelet reactivity was associated with differential interferon (IFN)-γ and interleukin (IL)-1β/IL-6 cytokine responses in whole blood and peripheral blood mononuclear cells (PBMCs). Through a series of *ex vivo* experiments, we determined that while the addition of isolated washed platelets

downregulated IFN-γ and upregulated IL-1β/IL-6 cytokine responses of PBMCs to several bacterial ligands, recombinant IFN-g and IL-1β/IL-6 had no effect on platelet function. We also found that the genetic variations which determine platelet function had overlapping associations with cytokine responses to different ligands. Although some of these variants are located in gene regions previously associated with immune properties or responses, others are known to be exclusively associated with platelet function. *Vice versa*, genetic variations determining cytokine responses to several ligands were also found to be associated with platelet reactivity. Our data suggest that platelets play an important role as cellular mediators in the crosstalk between haemostasis and inflammation, and indicate platelets as having a shared genetic regulatory mechanism with inflammation.

Ex vivo studies have reported that clopidogrel reduces pro-inflammatory responses to TLR4 stimulation, (13) but increases pro-inflammatory response upon TLR2 stimulation (14). We hypothesized that differences in platelet responses and platelet-monocyte complex (PMC) formation to TLR2 and TLR4 ligands influence the inflammatory response by leukocytes and that this also explains the seemingly opposite effects of $P2Y_{12}$ inhibitors on the inflammatory responses in the aforementioned studies. In **chapter 3**, we described through a series of *ex vivo* studies, that platelets attenuated the production of pro-inflammatory cytokines following PBMC stimulation with the TLR2/TLR1 ligand Pam3CSK4, whereas they increase the production of these cytokines following stimulation with the TLR4 ligand LPS. These effects of platelets are dependent on direct PMC formation and for the Pam3CSK4-induced response, on phagocytosis of platelets by monocytes. In line with these results, in our *ex vivo* study, healthy volunteers receiving ticagrelor showed increased pro-inflammatory cytokines in whole blood stimulated *ex vivo* with Pam3CSK4, while showing decreased cytokines in LPS-stimulated blood. Data from clinical studies in humans on the effects of $P2Y_{12}$ receptor antagonists on the susceptibility and outcome of infections are limited, if not somewhat conflicting. On one hand, clopidogrel has been reported to increase the risk for surgical site infections and bacteremia following coronary artery bypass surgery (15), as well as community acquired pneumonia (CAP), while on the other hand its use was also associated with a more favourable outcome in pneumonia and sepsis (16, 17). Our findings are of potential clinical relevance as the use of platelet inhibitors may alter the immune response during infections while the attenuation or augmentation of this response may depend on the type of microorganism and TLRs involved.

Modulation of platelet function during systemic inflammation

Platelets can complicate the systemic inflammatory response as excessive platelet activation may lead to the formation of microthrombi, release of proteins with immune modulatory properties and interaction with leukocytes leading to their activation (18).

In sepsis, the therapeutic strategy may be directed at intervening with the dysregulated response of platelets during systemic inflammation (19). Acute kidney injury (AKI) often develops in severe sepsis and platelets may play an etiological role in this complication (18, 20). The human recombinant alkaline phosphatase (recAP) is a novel dephosphorylating enzyme currently under investigation as a new therapy for sepsis-associated AKI due to its dephosphorylating effect on LPS and adenosine diphosphate (ADP). Bovine-derived alkaline phosphatase had previously been reported to inhibit platelet activation, but the precise mechanisms remained only partly understood. In **chapter 4,** we described the platelet-inhibiting action of recAP and its mechanisms of action through a series of *ex vivo* experiments in healthy volunteers and sepsis patients. We demonstrate that recAP strongly inhibits ADP-induced platelet activation in whole blood and cross-linked collagen-related peptide (CRP-XL)-induced platelet activation in platelet-rich plasma. This study reveals a previously undescribed mechanism of how recAP inhibits platelet function. The dephosphorylation of ADP, which is the most potent activator of the $P2Y_{12}$ receptor and amplifier of the platelet response, and the formation of adenosine as its turnover product are primarily responsible for the observed platelet-inhibiting effects. Additionally, exposure of whole blood of patients with septic shock to recAP reversed the sepsis-associated platelet hyperreactivity. This platelet-inhibiting effect may contribute to the therapeutic potential of recAP as a new candidate to treat sepsis-induced AKI. In addition, our findings support the idea that further studies are needed to investigate the effects of recAP treatment in other diseases, in which platelet-activation plays a relevant role.

Systemic inflammation, which may lead to the activation of platelets and coagulation, commonly co-occur with hypoxia in sepsis patients. Hypoxia may arise in sepsis due to enhanced metabolic demands, and decreased oxygen delivery (4). Both systemic inflammation and hypoxia were associated with adverse outcome in sepsis patients. Hypoxia had been implied to increase platelet reactivity in rats (21) and activate coagulation (22), but *in vivo* data in humans were largely missing. In **chapter 5**, we studied the effect of hypoxia during human endotoxemia on platelet activation. Our data showed that systemic hypoxia during systemic inflammation in humans *in vivo* does not augment, but rather attenuates, endotoxemia-induced platelet-monocyte complex formation and platelet hyperreactivity. This platelet-attenuating effect of hypoxia may be explained by lower levels of circulating pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF)-α, IL-6 and IL-8 in these subjects. Alternatively, hypoxia might result in increased levels of prostaglandin I_2 (PGI_2), which is known to inhibit platelet activation (20). Our study has limitations regarding the experimental human endotoxemia model used, which does not fully represent the inflammatory response observed in critically ill sepsis patients. However, given the paucity of human *in vivo* data on the effect of hypoxia on platelet reactivity, it provides valuable insights into the

complex interactions between inflammation, coagulation, and hypoxia.

Infections alter platelet function

Increased platelet activation and reactivity during infections

Acute bacterial infections are associated with an increased risk for cardiovascular events (CVE), but the magnitude of this risk depends on the type of infections (23-25). Clinical studies have shown a high incidence of cardiovascular complications in patients with Gram-positive bacterial infection (26, 27). Additionally, community acquired pneumonia (CAP), which is frequently caused by the Gram-positive bacteria *Streptococcus pneumoniae*, is associated with an increased short-term and long-term risk for CVE. Such risks appears lower for urinary tract infections, which are usually caused by *Escherichia coli* or other Gram-negative bacteria (23, 28). In addition, infective endocarditis is predominantly caused by Gram-positive bacteria (29). Platelets play a central role in acute CVE and atherosclerosis, but whether systemic platelet activation and platelet hyperreactivity indeed occur *in vivo* during Gram-positive bacterial infection, or invasive pneumococcal infection in particular, is not well-established.

In **chapter 6**, using a novel porcine model of pneumococcal disease we demonstrated that invasive *S. pneumoniae* infections induce pronounced *in vivo* platelet activation and hyperreactivity. Direct stimulation of platelets by *S. pneumoniae* mediated these effects, at least in part, as porcine washed platelets were activated by *ex vivo* exposure to *S. pneumoniae*. Pneumococci can activate platelets directly through different processes, including binding to platelet TLR2, FcγRIIA and the integrin αIIbβ3 (30), or activation of the platelet activation factor (PAF) receptor (31).

The study of pneumococcal pathogenicity also benefits from advances in bacterial genome sequencing. This technique, for example, has allowed for the prediction of which bacterial isolates tend to cause severe disease from their genome sequence alone (32, 33). In **chapter 7**, we sequenced 349 clinical isolates from patients with invasive pneumococcal disease and found that the phage-derived gene *pblB* was associated with death within 30 days of hospitalization. PblB of *Streptococcus mitis* was shown to function in the bacterial adhesion to platelets by interacting with α 2-8-linked sialic acid residues on platelet membrane gangliosides (34). PblB expression of *S. mitis* was also found to contribute to virulence in an *in vivo* rabbit model of infective endocarditis. This study indicated that PblB may be important for platelet deposition onto the infected valve surface, and the resultant formation of macroscopic vegetations (35)(16). More recently, Hsieh and colleagues showed that *pblB* knock-out mutant pneumococci had decreased adherence to respiratory epithelial cells and platelets (36). Through a series of *ex vivo* experiments, we found that fluoroquinolones, an antibiotic that is frequently

used in our Dutch patient cohort as empiric therapy for severe CAP together with penicillin, induced *pblB* expression. Finally, we found that wild type, *pblB*-expressing pneumococci induced higher platelet P-selectin expression, platelet-fibrinogen binding and PMC formation in whole blood when compared to the *pblB* knock-out mutant, irrespective of antibiotics exposure. This may explain why bacteremic patients, infected with pneumococci containing the *pblB* gene, have a higher chance to die within 30 days. An approximate of 20% increase from baseline values in platelet P-selectin expression and PMC has been associated with adverse cardiovascular events and the acute phase of ischaemic stroke (9, 37), and the increase in platelet activation associated with *pblB* in our *ex vivo* experiments exceeded this aforementioned value. This study also shows the value of integrating extensive bacterial genomics and clinical data in predicting and understanding pathogen virulence, which in turn will help to improve prognosis and therapy.

As mentioned previously, in chapter 3 we found that *ex vivo*, the TLR2 synthetic ligand Pam3CSK4 strongly induced PMC formation whereas LPS, a synthetic ligand of TLR4 did not. TLR2 and TLR4 ligations represent the immunological recognition against Gram-positive and Gram-negative bacteria, respectively (38). We speculated that the higher risk for CVE in Gram-positive bacterial infection compared to the Gram-negative bacterial infection (26-28) can be explained by differences in the platelet function of these patient groups. In **chapter 8**, we studied platelet reactivity, PMC formation and activation of the plasmatic coagulation in a cohort of patients with either Gram-positive or Gram-negative sepsis. We found that sepsis with common Gram-positive pathogens is associated with more pronounced platelet activation, platelet hyperreactivity and PMC formation compared with sepsis due to common Gram-negative pathogens. The majority of patients in the Gram-positive group had a *S. aureus* sepsis, whereas *E. coli* was the most common bacteria in the Gram-negative group. *S. aureus* and *Streptococcus spp.* are able to directly bind and activate platelets and different mechanisms through which these pathogens activate platelets have been reviewed recently by Hamzeh-Cognasse et. al. (39). *E. coli* was also recently demonstrated to activate platelets in a FcγRIIa-dependent manner (40, 41), although *ex vivo* data of platelet activation by *E. coli* LPS are conflicting (42-45). Our data of limited platelet activation in patients with *E. coli* sepsis are in line with reports that LPS administration to healthy volunteers does not appear to significantly activate platelets (46, 47). This study was limited by the relatively small number of patients in each group and the fact that the platelet reactivity assay was only performed once after the blood cultures became positive. Additionally, our results cannot be extrapolated to other Gram-positive and Gram-negative bacteria not included in our study, as the platelet-activating properties of these bacteria may differ. However, this study provides the evidence that Gram-positive sepsis is associated with marked platelet activation, in contrast to Gram-negative sepsis, and this may contribute to the

vascular complications seen in these infections.
These findings support the case for platelet function inhibitors as a preventive intervention to reduce acute vascular complications during invasive pneumococcal disease and other Gram-positive bacterial infections. A small placebo controlled trial involving 185 patients found that aspirin reduced the occurrence of CVE in CAP patients from 10.6% to 1.1% (48). In addition, a large observational study involving over 1000 elderly CAP patients reported lower 30-day mortality rate for those who had been on chronic treatment with aspirin, compared to those who had not (49). Larger placebo-controlled studies are warranted to provide evidence-based recommendations.
Another infection with an increased risk of cardiovascular diseases (CVD) (50) is HIV. HIV is has been associated with platelet activation (51). Intensification of antiretroviral therapy (ART) with the integrase inhibitor raltegravir (RAL) was reported to suppress residual viral replication and reduce plasma levels of the coagulation marker D-dimer (52). The switch from a protease inhibitor or enfuvirtide to RAL also decreased inflammatory and coagulation biomarkers (53). We therefore hypothesized that virologically suppressed HIV-infected patients using a RAL-based regimen have reduced platelet reactivity and PMC formation compared with those using a nonnucleoside reverse transcriptase reverse inhibitor (NNRTI)-based or protease inhibitor-based regimen. In **chapter 9**, we describe that HIV-infected individuals on a RAL-based regimen show reduced platelet hyperreactivity and PMA compared with those on an NNRTI or a protease inhibitor based regimen. Our findings are clinically relevant as higher values of these parameters are associated with an increased risk for future cardiovascular events (54, 55). Whether reduction in platelet reactivity and PMC in RAL-treated individuals are indeed associated with reduced cardiovascular complications requires studies with longer follow-up periods. However, due to its near absence of adverse effects on glucose and lipid metabolism (56), RAL is among the preferred ART in patients with a high cardiovascular risk. Our findings provide an extra argument to consider a RAL-based regimen in these high-risk patients.

Platelet dysfunction and bleeding complications during infections
Infections may not only lead to platelet hyperreactivity, but also platelet dysfunction, such as during dengue virus infection (57). Severe leptospirosis, a disease caused by the pathogenic *Leptospira spp.*, is often complicated by bleeding complications, of which the pathogenesis is still largely unknown. We hypothesized that the hemorrhagic complications are not only related to thrombocytopenia, but also to platelet dysfunction, and that increased binding of von Willebrand factor (VWF) to platelets is involved in both platelet dysfunction and increased platelet clearance. We described in **chapter 10** a prospective study in Semarang, Indonesia, enrolling 33 hospitalized patients with leptospirosis, of whom 15 developed clinical bleeding, and 25 healthy controls. We

measured platelet reactivity, platelet-VWF binding and assessed bleeding complications using the WHO bleeding scale. Our study reveals four important findings. First, platelet activation is a feature of leptospirosis with bleeding manifestations. Second, circulating platelets in leptospirosis patients express more VWF and this has a strong negative association with platelet number, in contrast with the plasmatic coagulation marker TAT complex. Third, VWF binding to platelets, mediated by the platelet GPIb, was associated with platelet activation, but not with platelet dysfunction. Four, bleeding complications are predominantly associated with platelet dysfunction rather than absolute platelet count. Platelet activation in leptospirosis may occur due to TLR4 and TLR2 ligation by *Leptospira spp.*, the latter having a stronger platelet-activating properties, or increased platelet-VWF binding. We also speculate that excessive platelet activation with secondary platelet 'exhaustion' underlies the observed platelet dysfunction, as previously found in patients with dengue (57). Increased platelet-VWF binding may play a role in increased platelet clearance. Platelet-VWF binding results in a structural unfolding of the GPIbα extracellular domain and triggers signaling into the platelet, desialylation of the platelet surface and increased uptake of platelets by the Ashwell-Morell receptor (58).

Considerations about the clinical implications of our findings: the use of platelet function inhibitors during inflammation and infection

Platelet hyperreactivity increases the risk for thrombotic vascular disease and may affect the inflammatory response at the same time. On the other hand, reduced platelet function during inflammation/infection may not only lead to bleeding complications but also lower the effect of platelets on the inflammatory response. Platelet inhibition has been suggested to have beneficial effects during sepsis as platelets contribute to platelet-leukocyte complex formation, microvascular thrombosis, tissue hypoxia and eventually organ failure. Depending on the stimulus, platelet-leukocyte interaction may have different effects. It may result in a pro-inflammatory phenotype, accelerating organ injury (18) or inhibition of the release of inflammatory mediators, resulting in an anti-inflammatory phenotype (59). Anti-platelet therapy may therefore influence inflammation, either directly or via platelet-leukocyte complex formation. The cyclooxygenase inhibitor aspirin and $P2Y_{12}$ receptor antagonists are the therapy of choice for prevention of thrombotic vascular events. All these drugs influence inflammation and the drugs in the latter group, clopidogrel and prasugrel, have also been found to inhibit the interaction between platelets and leukocytes and as such attenuate inflammation (60, 61). In line with this is our finding (chapter 3) that the administration of $P2Y_{12}$ receptor antagonist ticagrelor in healthy volunteers led to *ex vivo* inhibition of platelet-monocyte complex formation and subsequent reduction in proinflammatory cytokine

response after TLR4 stimulation.

Apart from its effects on inflammation, platelets can also play a more direct role in the innate and adaptive response against micro-organisms. Platelets are for instance able to enhance the phagocytosis of bacteria by neutrophils (62) or trigger neutrophil extracellular trap formation (63). The question is therefore whether and how platelet function inhibitors influence the host response to infectious diseases. A limited number of studies are available, sometimes with seemingly conflicting results. A retrospective cohort study of 1677 patients undergoing coronary artery bypass surgery showed that the preoperative use of aspirin plus clopidogrel increased the 30-day incidence of postoperative surgical site infection and bacteremia when compared with aspirin alone (15). This clinical study showed that suppression of platelet function was associated with the vulnerability to infection and bacteremia. Results of the PLATelet inhibition and patient Outcomes (PLATO) trial showed an opposite effect in patients with sepsis and critical illness. The lower overall mortality in the PLATO patients treated with ticagrelor compared to clopidogrel was partly the result of a lower death rate from pulmonary adverse events and sepsis (64). In patients with sepsis, aspirin was found to reduce mortality (65) and incidence of acute lung injury (ALI) (66, 67), while another study did not find its association with ALI (68). One study reported that although clopidogrel reduced the severity of sepsis, it increased the incidence of CAP (17). We hypothesized, based on the results of our studies, that these differences in outcomes may be due to the differences in the causative pathogens involved. Our *ex vivo* study (chapter 3) demonstrated namely that platelets have opposing inflammatory effects on cytokine response of PBMC depending on the TLR stimulation involved. The patient study described in chapter 8 also showed differences in platelet reactivity as only patients with a Gram-positive sepsis demonstrated increased platelet reactivity and PMC formation in contrast to those with Gram-negative sepsis. This may also be an underlying factor of why Gram positive bacteremia is associated with a higher risk for vascular complications and infective endocarditis (27, 29, 69, 70).

The inhibition of platelet function may therefore be of value to prevent vascular complications after infections caused by this group of pathogens. The benefit of prophylaxis should however be weighted against the risk of bleeding and attenuation of the inflammatory response. Furthermore, it should also be considered whether aspirin is used and at what dosage or whether $P2Y_{12}$ inhibitors are preferred, alone or in combination with aspirin. Patients with Gram-positive bacterial infections possibly benefit the most from adjunctive $P2Y_{12}$ inhibition, due to their concomitant inhibition of platelet-leukocyte complex formation, but this should first be investigated in an appropriate model. Our novel porcine model of pneumococcal disease, as described in chapter 6, could provide insight on the beneficial or detrimental effects of the various anti-platelet drugs. It is thereby important to consider which strain of pneumococcus is

used, as was demonstrated in chapter 7. Finally apart from aspirin and $P2Y_{12}$ inhibitors, the use of other agents that may inhibit platelet function, such as recAP (chapter 4), needs to be studied in infectious disease and inflammatory conditions with increased platelet activation.

Platelet hyperreactivity is not only seen in Gram-positive bacterial infection but also in viral infections such as HIV (chapter 9). Aspirin is adviced for the primary prevention of thrombotic vascular disease in HIV-infected patients based on the age-stratified 10-year coronary heart disease (CHD) risk score for men and the 10-year stroke risk score for women (71). The use of $P2Y_{12}$ inhibitors is generally not considered in the current guidelines. Cytochrome P450 interaction may have limited the use of $P2Y_{12}$ inhibitors in treated HIV infected subjects so far, but the guidelines can be reconsidered with the recent introduction of integrase inhibitors that do not affect cytochrome P450 activity. HIV is characterized by persistent inflammation with increased risk for thrombotic vascular events as a result. Consequently, the effect of $P2Y_{12}$ inhibitors is possibly more favorable compared to aspirin as it is a better inhibitor of platelet-leukocyte interaction.

Infectious diseases may not only result in platelet hyperreactivity, but may lead to platelet dysfunction as well. Researchers from our group have recently described this in dengue virus infections (57) and a similar phenomenon is described for leptospirosis in chapter 10. Our finding that platelet-WVF complex formation was associated with thrombocytopenia suggests that inhibition of the platelet-VWF interaction may be considered as a therapeutic option in patients with leptospirosis. Two newly developed drugs should be studied for that purpose: anti–VWF humanized single-variable-domain immunoglobulin (caplacizumab) has been developed that inhibits the GPIb binding site of VWF (72) or an antagonist of the platelet GP1b receptor, anfibatide (73). These drug have been developed as an anti-thrombotic therapy while haemorhage is a main complication of a severe leptospirosis infection. However, they may have a favorable effect in leptospirosis as our data suggest that excess platelet-VWF complex formation play a role in its pathogenesis. This concept should be thoroughly tested in an animal model before being tried in a clinical setting.

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Appendix

Summary

Platelets are traditionally known for their role in hemostasis. Mounting evidence also highlights their novel role as key effector cells of inflammation and host defense. Platelets interact with professional immune cells and play a major role in atherosclerosis and acute cardiovascular events (CVE). Thus, on the one hand, platelets may modulate the immune response and pathogenesis of infectious diseases, whereas on the other hand, inflammation and infections may affect platelet numbers and function.

Chapter 1 contains a general introduction to the physiology of platelets and their role as effector cells of inflammation and immunity. It also describes the background of the flow cytometry-based platelet function assays that were used in this thesis. Finally, we described the background of studies on platelet reactivity that we performed in both healthy volunteers and patients with infectious diseases.

Despite findings that platelet numbers and function influence inflammation and immune response and *vice versa*, the mechanisms of these interactions have not been well-established. In **chapter 2**, as part of the Human Functional Genomics Project (HFGP), we related platelet number and platelet reactivity to inflammatory markers and cytokine responses of whole blood and peripheral blood mononuclear cells (PBMCs) in the 500-Human Functional Genomics (500FG). This cohort consisted of approximately 500 healthy, Caucasian individuals. Our main findings were that platelet numbers were positively associated with circulating IL-1β levels and that platelet reactivity was associated with differential interferon (IFN)-γ and interleukin (IL)-1β/IL-6 cytokine responses. We found that genetic variations in platelet function had overlapping associations with cytokine responses to multiple ligands. *Vice versa*, genetic variations determining cytokine responses were also found to be associated with platelet reactivity.

The effects of platelets on immune responses may depend on the immune stimulus. In **chapter 3**, we described through a series of *ex vivo* studies, that platelets attenuate the production of pro-inflammatory cytokines following PBMC stimulation with the Toll-like receptor (TLR)2/TLR1 ligand Pam3CSK4, whereas they increase the production of these cytokines following stimulation with the TLR4 ligand lipopolysaccharide (LPS). These effects of platelets are dependent on direct platelet-monocyte complex (PMC) formation and for the Pam3CSK4-induced response, on phagocytosis of platelets by monocytes. In line with these results, healthy volunteers receiving the $P2Y_{12}$ receptor antagonist ticagrelor showed increased pro-inflammatory cytokines in whole blood stimulated *ex vivo* with Pam3CSK4, while showing decreased cytokines in LPS-stimulated blood. Our findings are of potential clinical relevance as the use of platelet inhibitors may thus alter the immune response during infections. The direction of this alterations will depend on the type of micro-organism and the TLRs involved.

Excessive platelet activation plays a role in the pathogenesis of sepsis. Adjunctive therapies aimed at platelets may improve the outcome of sepsis. Recombinant alkaline phosphatase (recAP) is a dephosphorylating enzyme that is currently under clinical investigation as a new therapy for sepsis-associated acute-kidney injury (AKI). How recAP modulates sepsis-associated organ failure is not well established. **Chapter 4** describes experiments using blood of healthy volunteers and sepsis patients showing that recAP inhibits platelet activation. RecAP promotes the dephosphorylation of ADP, which is the most potent activator of the $P2Y_{12}$ receptor and amplifier of the platelet response, and the formation of adenosine as its turnover product. This platelet-inhibiting effect may contribute to the therapeutic potential of recAP as a new candidate to prevent sepsis-induced AKI.
In **chapter 5**, the effect of hypoxia on platelet activation and PMC formation during human endotoxemia is studied. The data show that systemic hypoxia during systemic inflammation in humans *in vivo* does not augment, but rather attenuates, endotoxemia-induced PMC formation and platelet hyperreactivity. Given the paucity of human *in vivo* data on the effect of hypoxia on platelet reactivity, our study provides valuable insights into the complex interactions between inflammation, coagulation, and hypoxia.
The risk for vascular complications differs across bacterial infections. In general, vascular and thrombotic complications appear to be more common in infections caused by Gram-positive bacteria than in Gram-negative bacteria. Infections with the Gram-positive bacteria *Streptococcus pneumoniae* are well known for their cardiovascular complications. We demonstrated in **chapter 6**, using a novel porcine model of pneumococcal disease, that invasive *S. pneumoniae* infections induce pronounced *in vivo* platelet activation and hyperreactivity. Direct stimulation of platelets by *S. pneumoniae* mediated these effects, at least in part, as porcine washed platelets were activated by *ex vivo* exposure to *S. pneumoniae*. In **chapter 7**, we sequenced 349 pneumococcal clinical isolates from patients with invasive pneumococcal disease and found that the phage-derived gene *pblB* was associated with death within 30 days of hospitalization. Previously, PblB of *Streptococcus mitis* was shown to function in the bacterial adhesion to platelets and contribute to virulence in an *in vivo* rabbit model of infective endocarditis. We further found that wild type, *pblB*-expressing pneumococci induced higher platelet P-selectin expression, platelet-fibrinogen binding and PMC formation in whole blood when compared to the *pblB* knock-out mutant, irrespective of antibiotics exposure. This may explain why bacteremic patients, infected with pneumococci containing the *pblB* gene, have a higher chance to die within 30 days.
In **chapter 8**, results from an observational study are described, showing that sepsis with common Gram-positive pathogens is associated with more pronounced platelet activation, platelet hyperreactivity and PMC formation compared with sepsis due to common Gram-negative pathogens. This may explain why vascular complications are more common in Gram-positive bacterial infections.

Another infection that is associated with an increased risk of cardiovascular diseases (CVD) is HIV. Persistent platelet activation despite the use of antiretroviral therapy (ART) has been described. In **chapter 9**, we show that HIV-infected individuals on a raltegravir-based regimen had reduced platelet hyperreactivity and platelet-monocyte aggregates compared with those on a nonnucleoside reverse transcriptase inhibitor-based or protease inhibitor-based regimen.

Infections may not only lead to platelet hyperreactivity, but also platelet dysfunction, and this may lead to a bleeding tendency. In **chapter 10,** a prospective study is described enrolling patients with severe leptospirosis in Semarang, Indonesia. Of the 33 hospitalized patients, 15 developed clinical bleeding. We found increased platelet activation in leptospirosis patients who developed bleeding manifestations. This group of patients also showed more binding of von-Willebrand factor (VWF) to the platelet glycoprotein (GP)Ib, and this was negatively associated with platelet number. This suggests that increased platelet-VWF binding plays a role in increased platelet clearance, possibly through glycan structural changes leading to the increased uptake of platelets by the hepatic Ashwell-Morell receptor. Interestingly, the bleeding complications in patients were predominantly associated with platelet dysfunction rather than absolute platelet number. Our findings suggest that excessive platelet activation with secondary platelet 'exhaustion' underlies the observed platelet dysfunction, as previously found in patients with dengue.

Ringkasan Bahasa Indonesia

Platelet secara tradisional diketahui berperan dalam hemostasis. Bukti-bukti juga menunjukkan peran baru mereka sebagai sel efektor kunci dalam inflamasi dan pertahanan pejamu. Platelet berinteraksi dengan sel imun profesional dan memainkan peran besar dalam aterosklerosis dan kejadian kardiovaskuler akut. Di satu sisi, platelet dapat memodulasi respon imun dan patogenesis penyakit infeksi, sedangkan di sisi lain, inflamasi dan infeksi dapat mempengaruhi jumlah dan fungsi platelet.

Bab 1 memuat pengantar umum fisiologi dari platelet dan peran mereka sebagai sel efektor dari inflamasi dan imunitas. Bab ini juga menjelaskan latar belakang tes fungsi platelet berbasis *flow cytometry* yang kami gunakan dalam tesis ini. Kami juga menjelaskan latar belakang dari penelitian reaktivitas platelet yang kami lakukan pada sukarelawan sehat dan pasien penyakit infeksi.

Walaupun terdapat temuan-temuan bahwa jumlah dan fungsi platelet mempengaruhi inflamasi serta respon imun dan sebaliknya, mekanisme interaksi ini belum diketahui secara pasti. Dalam **bab 2**, sebagai bagian dari *Human Functional Genomics Project (HFGP)*, kami menghubungkan jumlah dan reaktivitas platelet dengan penanda inflamasi dan respon sitokin dari darah dan sel mononuklear darah tepi/ *peripheral blood mononuclear cells (PBMCs)* dalam *500-Human Functional Genomics (500FG)*. Kohort ini terdiri dari sekitar 500 individu Kaukasia sehat. Temuan utama kami adalah bahwa jumlah platelet terasosiasi dengan respon sitokin interferon (IFN)-γ dan interleukin (IL)-1β/IL-6 yang berbeda. Kami juga menemukan bahwa variasi genetik dari fungsi platelet juga terasosiasi dengan respon sitokin terhadap berbagai ligan. Sebaliknya, variasi genetik yang menentukan respon sitokin juga terasosiasi dengan reaktivitas platelet.

Efek dari platelet terhadap respon imun dapat bergantung pada stimulus imun yang terlibat. Dalam **bab 3**, kami menjelaskan melalui penelitian *ex vivo*, bahwa platelet menekan produksi sitokin pro-inflamasi setelah stimulasi *PBMC* dengan ligan *Toll-like receptor (TLR)2/TLR1* Pam3CSK4. Di lain pihak platelet meningkatkan produksi sitokin-sitokin ini setelah stimulasi dengan ligan *TLR4* yaitu lipopolisakarida (LPS). Efek platelet ini bergantung pada formasi kompleks platelet-monosit, sedangkan respon yang diinduksi oleh Pam3CSK4 bergantung pada fagositosis platelet oleh monosit. Sejalan dengan hasil ini, sukarelawan sehat yang diberi antagonis reseptor $P2Y_{12}$, ticagrelor, menunjukkan peningkatan sitokin pro-inflamasi di darah yang distimulasi *ex vivo* dengan Pam3CSK4, dan sebaliknya menunjukkan penurunan sitokin pada darah yang distimulasi LPS. Temuan kami berpotensi untuk memiliki relevansi klinis karena penggunaan inhibitor platelet dapat mengubah respon imun selama infeksi. Arah perubahan ini akan bergantung pada tipe mikroorganisme dan *TLR* yang terlibat.

Aktivasi platelet yang berlebihan memainkan peran dalam patogenesis sepsis. Terapi tambahan yang diarahkan terhadap platelet dapat memperbaiki luaran sepsis.

Recombinant alkaline phosphatase (recAP) adalah enzim defosforilasi yang sedang diteliti secara kinis sebagai terapi baru untuk cedera ginjal akut akibat sepsis. Bagaimana *recAP* dapat memodulasi gagal organ pada sepsis belum diketahui dengan pasti. **Bab 4** menjelaskan eksperimen menggunakan darah dari sukarelawan sehat dan pasien sepsis yang menunjukkan bahwa *recAP* menghambat aktivasi platelet. *RecAP* meningkatkan defosforilasi adenosin difosfat (ADP), yang merupakan aktivator dari reseptor $P2Y_{12}$ dan penguat respon platelet yang paling poten, dan meningkatkan formasi adenosin sebagai produk hasil degradasinya. Efek inhibisi terhadap platelet dari *recAP* dapat berkontribusi terhadap potensinya sebagai kandidat terapi untuk mencegah cedera ginjal akut yang diinduksi sepsis.

Dalam **bab 5**, kami membahas efek hipoksia terhadap aktivasi platelet dan formasi kompleks platelet-monosit selama endotoksemia manusia. Data kami menunjukkan bahwa hipoksia sistemik saat inflamasi sistemik pada manusia *in vivo* tidak meningkatkan, namun justru menurunkan formasi kompleks platelet-monosit dan hiperreaktivitas platelet. Karena sedikitnya data *in vivo* manusia mengenai efek hipoksia terhadap reaktivitas platelet, penelitian kami menghasilkan tilikan penting mengenai interaksi kompleks antara inflamasi, koagulasi dan hipoksia.

Risiko komplikasi vaskuler berbeda-beda tergantung dari jenis infeksi bakterial yang dialami. Secara umum, komplikasi vaskuler dan trombotik lebih umum terjadi pada infeksi yang disebabkan bakteri Gram positif dibanding bakteri Gram negatif. Infeksi akibat bakteri Gram-positif *Streptococcus pneumoniae* khususnya diketahui menimbulkan komplikasi kardiovaskuler. Kami menunjukkan dalam **bab 6**, dengan menggunakan binatang coba babi, bahwa infeksi invasif *S. pneumoniae* menginduksi peningkatan aktivasi platelet yang bermakna. Stimulasi langsung platelet oleh *S. pneumoniae* memerantarai sebagian efek ini karena platelet babi dapat teraktivasi oleh paparan *ex vivo* terhadap *S. pneumoniae*. Dalam **bab 7**, kami melakukan sekuensing genetik atas 349 isolat klinis pneumokokus dari pasien dengan penyakit pneumokokal invasif, dan menemukan bahwa gen *pblB* yang berasal dari phage terasosiasi dengan kematian yang terjadi dalam 30 hari sejak rawat inap dimulai. Sebelumnya, PblB dari *Streptococcus mitis* dilaporkan berperan dalam perlekatan bakteri ke platelet dan berkontribusi terhadap virulensi bakteri tersebut dalam sebuah model kelinci dari endokarditis infektif. Kami juga menemukan bahwa galur liar pneumokokus yang mengekspresikan *pblB* menginduksi ekspresi *P-selectin* platelet, ikatan platelet-fibrinogen dan formasi kompleks platelet-monosit yang lebih tinggi dalam darah dibandingkan dengan mutan *knock-out pblB*, terlepas dari adanya paparan terhadap antibiotik. Hal ini dapat menjelaskan mengapa pasien bakteremik yang terinfeksi dengan pneumokokus yang mengandung gen *pblB* berisiko lebih tinggi mengalami kematian dalam waktu 30 hari.

Dalam **bab 8**, kami memaparkan hasil studi observasional kami yang menunjukkan bahwa sepsis akibat patogen Gram positif terasosiasi dengan aktivasi platelet, hiperreaktivitas

platelet dan formasi platelet-monosit yang bermakna dibandingkan dengan sepsis akibat patogen Gram negatif. Hal ini dapat menjelaskan mengapa komplikasi vaskuler lebih banyak ditemui pada infeksi bakteri akibat Gram positif.

Infeksi lain yang terasosiasi dengan peningkatan risiko penyakit kardiovaskuler adalah HIV. Aktivasi platelet yang persisten tetap terjadi meskipun pasien menggunakan terapi anti-retroviral. Dalam **bab 9** kami menunjukkan bahwa individu terinfeksi HIV yang menggunakan regimen berbasis raltegravir mengalami penurunan hiperreaktivitas platelet dan agregat platelet-monosit dibanding mereka yang menggunakan regimen berbasis *nonnucleoside reverse transcriptase inhibitor* atau *protease inhibitor.*

Infeksi tidak hanya menimbulkan hiperreaktivitas platelet, namun juga disfungsi platelet dan hal ini dapat menyebabkan kecenderungan terjadinya perdarahan. Dalam **bab 10**, kami menjelaskan penelitian prospektif kami yang melibatkan pasien dengan leptospirosis berat di Semarang, Indonesia. Dari 33 pasien yang dirawat di rumah sakit, 15 mengalami perdarahan klinis. Kami menemukan peningkatan aktivasi platelet pada pasien leptospirosis yang mengalami manifestasi perdarahan. Kelompok pasien ini juga menunjukkan lebih banyak ikatan antara *von-Willebrand factor* (*VWF*) dengan *glycoprotein* (*GP*)*Ib* pada platelet, dan hal ini terasosiasi negatif dengan jumlah platelet. Hal ini menunjukkan bahwa peningkatan ikatan platelet-*VWF* memainkan peranan dalam meningkatkan penyingkiran platelet dari sirkulasi, kemungkinan melalui perubahan struktur glikan yang menyebabkan meningkatnya konsumsi platelet oleh reseptor Ashwell-Morell di hati. Menariknya, komplikasi perdarahan pada pasien lebih terasosiasi dengan disfungsi platelet dan bukannya dengan jumlah platelet. Temuan kami menunjukkan bahwa aktivasi platelet berlebih, dengan adanya kelelahan platelet sekunder, mendasari terjadinya disfungsi platelet sebagaimana yang sebelumnya telah ditemukan pada pasien dengue.

Nederlandse samenvatting

Bloedplaatjes (thrombocyten) zijn bekend om hun rol in de bloedstolling. Recent wetenschappelijk onderzoek laat echter zien dat zij ook belangrijk zijn in ontsteking en afweer. Thrombocyten interacteren intensief met witte bloedcellen en zijn betrokken bij atherosclerose en acute cardiovasculaire incidenten (CVI). Enerzijds moduleren thrombocyten dus de afweerreactie tegen infectieziekten, anderzijds kunnen infectieziekten en inflammatie de functie van thrombocyten beïnvloeden.

Hoofdstuk 1 geeft een algemene inleiding in de fysiologie van thrombocyten en hun rol in ontsteking en afweer. Het beschrijft de principes van de thrombocytenfunctietest op basis van flow cytometry die is gebruikt in verschillende hoofdstukken van dit proefschrift. Tenslotte wordt de achtergrond beschreven van de studies over veranderingen in bloedplaatjesfunctie die zijn uitgevoerd bij zowel gezonde vrijwilligers als patiënten met infectieziekten.

Er is nog veel onbekend hoe precies thrombocytenaantal en -functie de afweerrespons beïnvloeden en omgekeerd. **Hoofdstuk 2** beschrijft de associaties tussen thrombocytenaantal en functie enerzijds en inflammatiemarkers en cytokineresponsen anderzijds in een cohort van 500 gezonde mensen. Deze studie maakte deel uit van het Human Functional Genomics Project (HFGP). De belangrijkste bevindingen waren dat thrombocytenaantal positief geassocieerd is met circulerend interleukin (IL)-1β en dat thrombocytenreactiviteit positief geassocieerd is met IL-1β / IL-γ responsen en negatief met interferon(IFN)-g responsen. Genetische variatie in thrombocytenfunctie had meerdere overlappende associaties met cytokineresponsen en *vice versa*.

De effecten van thrombocyten op de immuunrespons is afhankelijk van de immuunstimulus. **Hoofdstuk 3** beschrijft dat thrombocyten de productie van pro-inflammatoire cytokines door leukocyten remmen na stimulatie met de Toll-like receptor (TLR)2/ TLR1 ligand Pam3CSK4, terwijl ze de productie van deze cytokines verhogen na stimulatie met de TLR4 ligand lipopolysaccharide (LPS). Deze effecten waren afhankelijk van de adhesie van thrombocyten aan leukocyten. Het remmend effect van thrombocyten op de cytokinerespons op Pam3CSK4 was daarnaast afhankelijk van fagocytose (opname) van thrombocyten door leukocyten. Deze bevindingen worden ondersteund door de observatie dat gezonde vrijwilligers die de thrombocytenremmer ticagrelor hadden ingenomen meer pro-inflammatoire cytokines maakten na stimulatie met Pam3CSK4, en minder cytokines na stimulatie met LPS. Het potentiële klinisch belang van deze observaties is dat thrombocytenremmers dus verschillende effecten kunnen hebben op de afweerrespons tegen infecties en dat deze effecten afhankelijk zijn van het type micro-organisme en de betrokken TLRs.

Overmatige thrombocytenactivatie speelt een rol in de pathogenese van sepsis. Nieuwe therapieën die aangrijpen op thrombocyten kunnen mogelijk de prognose van

sepsispatiënten verbeteren. Recombinant alkalisch fosfatase (recAP) is een defosforylerend enzym dat momenteel in een grote klinische trial onderzocht wordt als aanvullende therapie in sepsis. Een eerdere studie liet zien dat dit middel vooral het ontstaan van nierschade bij sepsis voorkomt. Hoe recAP deze orgaanschade voorkomt is nog niet volledig opgehelderd. **Hoofdstuk 4** beschrijft experimenten waarbij recAP de activatie van thrombocyten remt in bloed van gezonde vrijwilligers en sepsispatiënten. recAP stimuleert de defosforylering van ADP, een belangrijke activator van thrombocyten, en de vorming van adenosine, dat een plaatjesremmende functie heeft. Aangezien overmatige activatie van thrombocyten bijdraagt aan de orgaanschade tijdens sepsis, kunnen deze thrombocytremmende effecten van recAP bijdragen aan het voorkomen van nierschade in sepsis.

Hypoxie treedt vaak op bij sepsis. **Hoofdstuk 5** beschrijft het effect van hypoxie op thrombocytenactivatie en thrombocyt-monocyt interactie tijdens een humaan endotoxemie model, waarbij gezonde vrijwilligers LPS krijgen toegediend. Systemische hypoxie bleek de reactiviteit van thrombocyten en thrombocyt-monocyt interactie te remmen. Deze studie geeft zo inzicht in de complexe effecten van hypoxie op stolling en inflammatie bij de mens.

Het risico van vasculaire complicaties verschilt per bacteriële infectie. Vasculaire en trombotische complicaties komen in het algemeen vaker voor bij infecties veroorzaakt door Gram-positieve bacteriën dan bij Gram-negatieve bacteriën. De Gram-positieve bacterie *Streptococcus pneumoniae* (pneumokok) is een veelvoorkomende oorzaak van pneumonie en invasieve infecties en is relatief vaak geassocieerd met cardiovasculaire complicaties. **Hoofdstuk 6** beschrijft een nieuw ontwikkeld pneumokokken varkensmodel, waarin *S. pneumoniae* bacteriemie leidde tot een uitgesproken activatie van thrombocyten en de vorming van thrombocyt-monocytcomplexen. Directe stimulatie van thrombocyten door *S. pneumoniae* veroorzaakte deze effecten. **Hoofdstuk 7** beschrijft de resultaten van een studie waarin 349 pneumokokkenisolaten van patiënten met invasieve pneumokokkeninfecties zijn gesequenced. Aanwezigheid van het faag-afgeleide gen *pblB* bleek geassocieerd met een verhoogde kans op overlijden binnen 30 dagen na ziekenhuisopname. Eerder onderzoek liet zien dat *PblB* bij de bacterie *Streptococcus mitis* een rol had in de bacteriële adhesie aan thrombocyten en de virulentie van *S. mitis* in een konijnenmodel van endocarditis. Wildtype pneumokokken die *pblB* tot expressie brachten, induceerden meer activatie van thrombocyten dan pneumokokken zonder *pblB*, en dit effect was onafhankelijk van blootstelling aan antibiotica. We hypothetiseren dat dit effect op thrombocyten de hogere mortaliteit op 30 dagen verklaart.

Hoofdstuk 8 beschrijft de resultaten van een observationele studie waaruit blijkt dat thrombocyten van sepsispatiënten met vaak voorkomende Gram-positieve bacteriën aanzienlijk meer geactiveerd zijn dan thrombocyten van patienten met Gram-negatieve bacteriën. Thrombocyt-monocyt complexen waren ook meer toegenomen bij

Gram-positieve sepsis. Deze bevindingen kunnen mede verklaren waarom vasculaire complicaties vaker voorkomen bij Gram-positieve bacteriële infecties.
HIV is een andere infectie die geassocieerd is met een verhoogd risico op hart- en vaatziekten. HIV-geïnfecteerde personen hebben persistente thrombocytenactivatie, ondanks het gebruik van antiretrovirale therapie (ART). **Hoofdstuk 9** laat zien dat personen die een ART regime gebruiken dat gebaseerd is op de integrase remmer raltegravir, minder thrombocytenactivatie en thrombocyt-monocyt complexen hadden, vergeleken met hen die een regime met een nonnucleoside reverse transcriptase remmer of proteaseremmer gebruikten.
Infecties kunnen niet alleen leiden tot thrombocyten activatie en hyperreactiviteit, maar ook tot thrombocyten dysfunctie. Dit kan leiden tot een bloedingsneiging. **Hoofdstuk 10** beschrijft een prospectieve studie in patiënten met ernstige leptospirose in Semarang, Indonesië. Bijna de helft van deze patiënten hadden bloedingen. De bloedplaatjes van patiënten met ernstige leptospirose waren minder goed activeerbaar en dus dysfunctioneel. De mate van thrombocytdysfunctie was sterker geassocieerd met bloedingen dan het thrombocytenaantal. Von Willebrand factor (VWF) speelt een rol in de adhesie van thrombocyten aan de vaatwand en zorgt voor verwijderen van thrombocyten uit de circulatie. Thrombocyten van de leptospirosepatienten hadden meer VWF op hun membraan en dit was negatief gecorreleerd met de thrombocytenaantal. Deze gegevens dragen bij tot een beter inzicht in de oorzaak van de thrombocytopenie en bloedingscomplicaties bij leptospirose en de ontwikkeling van toekomstige therapeutische opties.

List of Publications

1. **Tunjungputri RN**, van der Ven AJ, Riksen N, Rongen G, Tacke S, van den Berg TN, Fijnheer R, Gomes ME, Dinarello CA, van de Veerdonk FL, Gasem M. Differential effects of platelets and platelet inhibition by ticagrelor on TLR2– and TLR4-mediated inflammatory responses. Thrombosis and haemostasis. 2015;113(5):1035-45.
2. **Tunjungputri RN**, Van Der Ven AJ, Schonsberg A, Mathan TS, Koopmans P, Roest M, Fijnheer R, Groot PG, de Mast Q. Reduced platelet hyperreactivity and platelet-monocyte aggregation in HIV-infected individuals receiving a raltegravir-based regimen. AIDS. 2014 Sep 10;28(14):2091-6.
3. **Tunjungputri RN**, de Jonge MI, de Greeff A, van Selm S, Buys H, Harders-Westerveen JF, Stockhofe-Zurwieden N, Urbanus RT, de Groot PG, Smith HE, van der Ven AJ. Invasive pneumococcal disease leads to activation and hyperreactivity of platelets. Thrombosis Research. 2016 Aug 31;144:123-6.
4. **Tunjungputri RN**, Peters E, van der Ven A, de Groot PG, de Mast Q, Pickkers P. Human recombinant alkaline phosphatase inhibits ex vivo platelet activation in humans. Thrombosis and Haemostasis. 2016;116(6):1111-21.
5. **Tunjungputri RN**, van de Heijden W, Urbanus RT, de Groot PG, van der Ven A, de Mast Q. Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis. Platelets. 2016 Dec 26:1-7.
6. **Tunjungputri RN**, Mobegi FM, Cremers AJ, van der Gaast-de CE, Ferwerda G, Meis JF, Roeleveld N, Bentley SD, Pastura AS, van Hijum SA, van der Ven AJ. Phage-Derived Protein Induces Increased Platelet Activation and Is Associated with Mortality in Patients with Invasive Pneumococcal Disease. mBio. 2017 Mar 8;8(1):e01984-16.
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8. Netea MG, Joosten LAB, Li Y, Kumar V, Oosting M, Smeekens S, Jaeger M, ter Horst R, Schirmer M, Vlamakis H, Notebaart R, Pavelka N, Aguirre-Gamboa R, Swertz MA, **Tunjungputri RN**, van de Heijden W, FranzosaEA, Ng EA, Graham D, Lassen K, Schraa K, Netea-Maier R, Smit J, de Mast Q, van de Veerdonk F, Kullberg BJ, Tack C, van de Munckhof I, Rutten J, van der Graaf J, Franke LL, Hofker M, Jonkers I, Platteel M, Maatman A, Fu J, Zhernakova A, van der Meer JWM, Dinarello CA, van der Ven A, Huttenhouwer C, Koenen H, Joosten I, Xavier RJ, Wijmenga C. Understanding human immune function using the resources from the Human Functional Genomics Project. Nature Medicine. 2016 Aug 1;22(8):831-3.

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Curriculum vitae

Rahajeng N. Tunjungputri, MD, also known as Ajeng, was born on 11 October 1984 in Jakarta, Indonesia. She spent her multicultural childhood in Indonesia across Kalimantan, Sumatra, and Java, as well as in the United Kingdom. After graduating with merits from high school, she received the invitation to undergo medical training by the Faculty of Medicine, Diponegoro University (FMDU) in Semarang, Central Java, Indonesia in 2002.

In the middle of her medical studies, she received a grant from Asia Europe Foundation for training on inter-cultural communication and volunteering program of UNESCO, the Asia Europe Young Volunteers Exchange (AEYVE), in France and Italy for 3 months. Later, during her medical internship in 2007, she was selected to join the course block of Infectious Disease in The Tropics in Radboudumc, Nijmegen, The Netherlands, under the supervision of dr. Monique Keuter, which triggered her deep interest in the subject and introduced her to the infectious disease group in Radboudumc.

After graduating as a medical doctor in early 2009, she worked as a general practitioner (dokter PTT) in a health center on the remote island of Matak, which is part of the Riau Islands province, off the shore of Sumatra.

In late 2009 she returned to Semarang and received her state civil service appointment as a lecturer in the Parasitology Department of FMDU in 2009. She performed research on molecular genetics in the Department of Human Genetics, Radboudumc in 2010-2011 as part of her master studies in Biomedical Sciences in FMDU.

In 2012, she was offered a PhD research position by Prof. Andre van der Ven in Radboudumc, with joint supervision by dr. Quirijn de Mast and Prof. M. Hussein Gasem in Semarang, and was awarded the Dikti-NESO PhD scholarship from the Indonesian Ministry of Education. She then performed her full time PhD research on platelets and infectious diseases in Nijmegen, The Netherlands, and undertook research projects in Jepara and Semarang, Indonesia, on leptospirosis and dengue. This led to the body of work you are currently reading.

During her PhD study, she presented her research at different international conferences, e.g. the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), in Amsterdam, The Netherlands, the International Leptospirosis Society (ILS) meeting in Semarang, Indonesia, and the Platelets International Symposium, in Wellesley, USA. She also presented at the Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH), in Philadelphia, USA, for which she received the 1st prize of the ASTMH Clinical Research Award in 2015.

She will continue her work as a clinician-researcher in FMDU as part of the Center for Tropical and Infectious Disease (CENTRID).

Next to continuing with her research work in clinical infectious diseases, she plans to pursue further clinical training as an internist and infectious disease specialist.